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Evaluation of the Tissue Culture Standard and Correlation with DNA Probes and ELISA for the Detection of Chlamydia trachomatis

Endocervical swabs collected in triplicate from 209 symptomatic and asymptomatic women in a high prevalence adolescent clinic (99) and in an intermediate prevalence University Health Service (110) were cultured simultaneously for isolation of Chlamydia trachomatis. Chlamydia was isolated from 33 patients, with 24 positive for all three specimens and nine with various patterns of positive and negative specimens. Discrepancy analysis consisting of multiple passes and reculture of discrepant negative specimens resolved swab-to-swab variation in 3/9 patients. This left 6/33 (18%) positive patients with discrepant negatives due to swab-to-swab variation. In the same populations, triplicate endocervical specimens were collected from 884 patients. Two DNA probes (non-isotopic (NIP) and isotopic (IP)) specific for chlamydial ribosomal RNA and an established ELISA were compared to the culture standard. There were no significant differences in the total sensitivities of the three non-culture tests compared to culture: NIP was 89.5%, IP 86.3%, and ELISA 90.5%. There was no difference between the false negative rates of the non-culture based techniques and the swab-to-swab variation in detection of culture itself. NIP specificity (93.2%) was significantly lower ($p<.05$) than those of IP (97.8%) and ELISA (98.2%). Discrepancy analysis of patient specimens in which non-culture test(s) did not agree with culture explained over 78% of the false negative and false positive non-culture results. Presently the IP and ELISA methods appear to be suitable diagnostic tests in these populations. The NIP which was in the final stage of development must reduce its false positive rate, but has reached a convenience and performance level near that of an established ELISA.

Title

Evaluation of the Tissue Culture Standard, and Correlation with DNA Probes and ELISA for the Detection of *Chlamydia trachomatis*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Pathology at the Medical College of Virginia, Virginia Commonwealth University

By

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Lastly and most importantly, I wish to express special gratitude to my family, especially to my wife for her help in typing this thesis, her encouragement, and loving patience during the last two years.

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INTRODUCTION

Genital infections caused by Chlamydia trachomatis are now the most prevalent and one of the most damaging of all the sexually transmitted diseases seen in the United States (162). C. trachomatis is the most prevalent sexually transmitted pathogen among adolescent and college age women. Complications of C. trachomatis infection of the female genital tract include: pelvic inflammatory disease, ectopic pregnancy, infertility, and vertical infection during parturition leading to neonatal pneumonia and conjunctivitis (162). Detection of C. trachomatis is, therefore, an indication for antimicrobial treatment of both asymptomatic and symptomatic young women (as well as their sexual contacts).

Tissue culture is the reference method for diagnosis of C. trachomatis infections (12,125,129). Tissue culture is, however, costly, time consuming, and unavailable in most clinical laboratories. Rapid antigen detection kits, including enzyme linked immunosorbent assay (ELISA) and direct fluorescent monoclonal antibodies (DFA) for direct detection in clinical specimens, have been available since 1984. These methods are usually less expensive, faster, and easier to perform than tissue culture; they have made

chlamydial testing more widely available. Until 1988, the use of nucleic acid probes for detection of C. trachomatis in the clinical laboratory was impractical. Gen-Probe, Inc. (San Diego, CA) has developed two rapid DNA probe-based assays (non-isotopic and isotopic) directed against the ribosomal RNA (rRNA) of C. trachomatis for detection of organisms directly from clinical specimens. The availability of these two different approaches to the nonculture based diagnosis of C. trachomatis infections led to increased interest in the comparative evaluation of these techniques.

Tissue culture is the method against which nonculture methods (antigen detection tests and nucleic acid probes) are measured (125,129). However, there is variation between the sensitivities of different culture methods; even when an optimal culture method is used, tissue culture is not 100% sensitive (125,129). When evaluating nonculture method(s) against the tissue culture standard, multiple swabs must be collected, normally one for each method. Therefore, when discrepancies arise between the tissue culture method and nonculture method(s), the question arises as to what portion of these discrepancies are due to swab-to-swab variation? A major drawback with the antigen detection methods has been the concern with false positive results (culture negative, antigen test positive results) (129). The question arises as to what portion of these false positive tests are true chlamydial infections (culture failures) and what portion

are real false positive reactions?

My research included two phases: phase (1), the culture variation study, was designed to evaluate the tissue culture method (the same procedure that would later be used to compare nonculture methods in phase (2)) and had the following objectives: (A) to determine the culture variation (swab-to-swab variability) when culturing endocervical specimens in triplicate from women in an adolescent clinic and in a university health service clinic; (B) to determine if the prevalence of C. trachomatis in a test population affects its recovery; (C) to determine if the order in which swabs are obtained from the endocervix influences detection of C. trachomatis; (D) to determine whether increasing the number of swabs cultured from the endocervix improves detection of C. trachomatis; and (E) to determine if the number of times a culture is "passed" significantly increases recovery of C. trachomatis; phase (2), the comparison of DNA probes and ELISA versus tissue culture had two objectives: (A) to compare two newly developed DNA probe-based assays and a commercial ELISA against a tissue culture reference standard for detection of C. trachomatis in the same two populations; and (B) to analyze in a systematic way the discrepancies between the culture standard and the rapid nonculture based techniques.

LITERATURE REVIEW

Historical Background

Trachoma, an infectious keratoconjunctivitis that leads to blindness, has been described since ancient times, with references in Egyptian papyri and Greco-Roman medical treatises (110). In 1907 Halberstaedter and Prowazek provided the first laboratory evidence for an infective cause of trachoma (110,120). They demonstrated that scrapings from a trachomatous eye could produce subclinical to mild conjunctivitis in orangutans, and that cytologic examination of conjunctival scrapings with Giemsa stain in both natural and experimental hosts revealed the presence of inclusion bodies (110). Identical inclusions were soon found in the conjunctivae of neonates demonstrating a non-gonococcal form of ophthalmia neonatorum, and in the cells of the genital tracts of some of their parents who had urethritis or cervicitis (120). This was followed by the discovery of an adult form of conjunctivitis similarly associated with genital tract infections (120).

The pandemic of psittacosis in 1929-30 prompted an effort to define the agents causing these infections in humans and psittacine birds (120). For the first time, cultural methods using mice and chick embryos were employed

and the causative agent of psittacosis was isolated from man and birds (110). The agent of lymphogranuloma venereum was isolated at about the same time (120,150). Further interest in this class of agents was raised in the 1950's when the occupational hazard of ornithosis from turkeys and other poultry was recognized (120).

A major advance occurred in 1957 when T'ang and co-workers isolated the etiological agent of trachoma in yolk sacs of embryonated hen eggs (110,120,150). Soon after, the organisms responsible for "amicrobial" ophthalmia neonatorum of the newborn and from infection of the genital tract of parents of children with ophthalmia were isolated (150). Identical organisms were also recovered in the genital tract of men presenting with non-gonococcal urethritis (150).

Although these intracellular infectious agents could now be grown, isolation in chick embryo yolk sacs was technically demanding and a slow procedure which could not be routinely used in clinical laboratories. The next major breakthrough occurred in 1965 when Gordon and Quan described a tissue culture procedure for the isolation of the agents causing trachoma and inclusion conjunctivitis (36,120). The tissue culture method also allowed for more extensive investigation of genital tract infections due to this class of agents now designated as Chlamydia (120) (see "Taxonomy" below).

Investigation of chlamydial diseases has continued to flourish (110,120,150). Since 1965 the tissue culture

procedure has improved, but it still has limited clinical availability. However, in the 1980's, a number of antigen detection tests became available making it possible for more clinical laboratories to test for Chlamydia directly from clinical specimens (23,135,154). Although perfect clinical diagnostic tests are not yet available (129), the clinical research tools are present that may lead to diagnostic tests that are both definitive and applicable in clinical laboratories.

Basic Characteristics

The chlamydiae are prokaryotic, gram-negative, non-motile, coccoid, obligate intracellular bacteria (92). These organisms are filterable and do not grow on conventional bacterial media. They were initially thought to be viruses, but their only similarities to viruses are that they are obligate intracellular parasites that are energy parasites (they require host ATP) (91). They differ from viruses because they possess both RNA and DNA, have cell walls similar in structure to gram-negative bacteria (although their cell wall has no peptidoglycan layer and lacks muramic acid) (91), are susceptible to certain antibiotics, possess a number of enzymes, undergo binary fission, and have a restricted metabolic capacity (12). The organism exists in two principal cell types (92): (1) elementary bodies, 0.2-0.4 um in diameter, are non-multiplying cells specialized for extracellular transit and entry into host cells; and (2) reticulate bodies (initial bodies), 0.6-1.5 um in diameter, are non-infectious cells specialized for intracellular multiplication (92). Chlamydia have a unique developmental cycle (see "Life Cycle" section) which differentiates them from all other micro-organisms (92,123). They replicate within the cytoplasm of host cells forming characteristic intracellular inclusions which can be stained and seen by light microscopy.

Taxonomy

Prior to 1966, the array of infectious agents now classified as Chlamydia were known by a variety of names. Because the organisms caused a wide range of diseases and could not be distinguished from each other by isolation and study in vitro, they were grouped into descriptive classifications such as TRIC agents (trachoma-inclusion conjunctivitis) that were, in turn, combined into loose grouping like the PLT (psittacosis-lymphogranuloma-trachoma) group of agents (150). Other important names for grouping of these organisms included Bedsonia and Miyagawanella (131). The chlamydiae are taxonomically separated from other obligate intracellular bacteria (the Rickettsiales), principally due to their unique life cycle, morphology, and fine structure (11,91,123). They are in their own order, Chlamydiales; presently the family chlamydiaceae comprises a single genus Chlamydia (92). The name Chlamydia comes from the greek word chlamys, meaning a cloak draped from the shoulder (150). The organisms were described in this manner due to the draping of intracytoplasmic inclusions around the nucleus of host cells.

The genus Chlamydia is divided into two species, Chlamydia trachomatis and Chlamydia psittaci. Both species share a common group antigen (3), however, there is less than 10% DNA homology between members of the two species (63). Table 1 lists four criteria that separate the two species (92). C. psittaci usually resides in birds and

mammals while C. trachomatis, except for several murine strains, is a human parasite. Both form cytoplasmic inclusions that are demonstrated by Giemsa stain, but the C. psittaci inclusion is larger and more diffuse than the C. trachomatis inclusion. C. trachomatis also synthesizes glycogen in its inclusions, in amounts detectable with Lugol's iodine, while C. psittaci does not. Lastly, C. trachomatis has a pathway for folate biosynthesis and, therefore, is sensitive to sulfadiazine; C. psittaci does not produce folate and is sulfadiazine resistant.

C. trachomatis is further divided into three biovars (92): (1) the mouse biovar, (2) the trachoma biovar, and (3) the lymphogranuloma venereum (LGV) biovar. Strains of the mouse biovar infect mice and cause mouse pneumonitis; they do not infect humans and have only 30-60% DNA homology with the trachoma biovar. Trachoma biovar (trachoma-inclusion conjunctivitis) has 12 identified serovars (A-K) which are strictly human pathogens with no animal reservoirs. These organisms infect columnar cells of mucous membranes. Centrifugation onto cell monolayers and/or treatment of cells with diethyl-aminoethyl (DEAE)-Dextran are needed for efficient infectivity of this biovar in tissue culture but are not needed for adequate isolation of the systemic LGV biovar. The serovars A, B, Ba, and C are associated with trachoma, whereas serovars D-K are associated with genital tract infections, inclusion conjunctivitis, and vertical transmission of disease to newborns. The lymphogranuloma

Table 1. Differentiation of the species of the genus *Chlamydia*[a]

Characteristics	*C. trachomatis*	*C. psittaci*
Natural hosts	Humans, mice	Birds, mammals other than human
Inclusion morphology		
oval, vacuolar	+	-
variable shape, dense	-	+
Glycogen inclusion	+	-
Folate biosynthesis	+	-

[a]Adapted from: Moulder, J.W. 1984. Order II. *Chlamydiales* Storz and Page 1971, 334[AL]. In Bergey's Manual of Systematic Bacteriology, Vol. 1. (Krieg, N.R. and J.G. Hold, Eds). Williams and Wilkins, Baltimore, page 735.

venereum biovar has three identified serovars, L1-L3. These strains are sexually transmitted pathogens that cause invasive rather than just mucosal infection. As with the trachoma biovar, there is no animal reservoir. The organisms preferred site of infection is lymph nodes, hence the name lymphogranuloma. The percent DNA homology of the LGV biovar with the trachoma biovar is 100%. However, the LGV and mouse biovars have characteristics *in vitro* that distinguish them from the trachoma biovar: when grown in cell culture using L-cells, they cause plaque formation; centrifugation of inoculum onto cell monolayer or pretreatment of cell monolayer with DEAE-Dextran does not enhance isolation in tissue culture. *In vivo* only organisms of the LGV biovar are lethal when they are inoculated intracerebrally into mice.

A Third Species?

Grayston *et al*. (37) reported a new *C. psittaci* strain designated TWAR as an important respiratory pathogen. Kuo *et al*. (68) characterized nine strains. Isolates grew poorly in tissue culture, but when they grew, intracytoplasmic inclusions that did not stain with iodine were present that were similar in morphology to *C. psittaci*. Based on immunological analysis using various *Chlamydia*-specific monoclonal antibodies, it was shown that the TWAR strains did belong to the genus *Chlamydia*, were distinct from *C. trachomatis*, but were also serologically unique among *C. psittaci*. Further investigation has shown that the

TWAR strains, unlike C. psittaci, are probably primary human pathogens (68). Campbell et al. (14) examined eight TWAR strains and reported that these strains did not contain plasmid DNA and that the strains could be readily distinguished from C. trachomatis and C. psittaci by restriction endonuclease analysis. They also reported that all their TWAR isolates had identical DNA homology by southern blot analysis but no significant homology with any other Chlamydia isolates. Lastly, Chi et al. (17) used transmission electron microscopy to examine the fine structure of strains of TWAR. TWAR elementary bodies demonstrated a pear-shaped morphology with a large periplasmic space different from other chlamydial organisms. The TWAR reticulate body was, however, morphologically and structurally similar to other Chlamydia species. Chi et al. also reported that TWAR undergoes the same developmental cycle as do other chlamydiae. Based on the above investigations, it does appear that the TWAR agents are definitively distinct from other known avian and mammalian C. psittaci isolates and are perhaps a separate species.

Life Cycle

As stated earlier (in "Basic Characteristics"), all chlamydiae share a developmental cycle seen nowhere else in nature (11,123). The chlamydial life cycle begins with the recognition and attachment of a metabolically inactive elementary body to an eukaryotic cell. The attachment process most likely involves specific receptor sites. The presence of the specific receptor probably determines which cell types are susceptible to infection. Wenman et al. (166) isolated surface proteins on the elementary body's surface not found on reticulate bodies that appear to bind to eukaryotic cell membranes. Penetration of chlamydiae into the host cell involves an active endocytosis induced by the chlamydiae with energy expended by the eukaryotic cell. The elementary body is enclosed within an endocytic vesicle (phagosome) of host cell origin (see figure 1). The chlamydiae specifically inhibit phagolysosomal fusion, preventing attack of the intracellular organisms by lytic lysosomal enzymes. One explanation for this ability is that a chlamydial surface antigen may inhibit fusion (123). Another possibility is that the chlamydiae may modify the phagosome membrane and this could be continually occurring as the phagosome grows (123). Within the phagosome differentiation occurs in approximately 6-8 hours when the elementary body changes to a metabolically active and dividing form called the reticulate body (see figure 2). This reorganization process is poorly understood. However,

it is known that chlamydiae use the host cells' ATP and pool of precursors to synthesize their own macromolecules such as RNA, DNA, and protein. Reticulate bodies begin multiplying by binary fission approximately eight hours after entry into the cell and continue until approximately 18-24 hours (see figure 3). Starting at approximately 18-24 hours, some of the reticulate bodies begin to reorganize back into smaller elementary bodies. As time goes by, the number of reticulate bodies converting to elementary bodies increases (see figure 4). The whole cycle takes place within the intracytoplasmic inclusion until inclusion membrane lysis occurs (between 24 and 48 hours) followed by host cell lysis releasing most of the elementary bodies to reinitiate the cycle. The chlamydial life cycle thus includes two morphological forms: (1) the compact and stable elementary body which can persist in the extracellular environment and is responsible for cell-to-cell and host-to-host transmission, and (2) the highly labile reticulate body which represents the metabolically active and vegetative form that is non-infective and cannot survive outside the host cell.

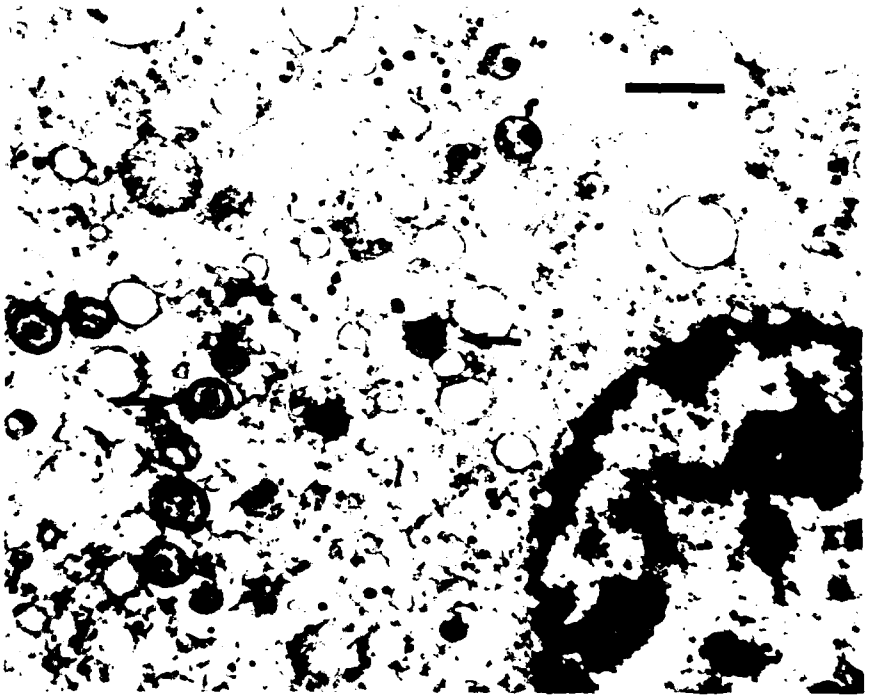

Figure 1. Transmission electromicrograph of elementary bodies after entering the McCoy cell by endocytosis at two hours post infection. Note the elementary bodies are beginning to differentiate into reticulate bodies (intermediate bodies). Bar = 1.0 um.

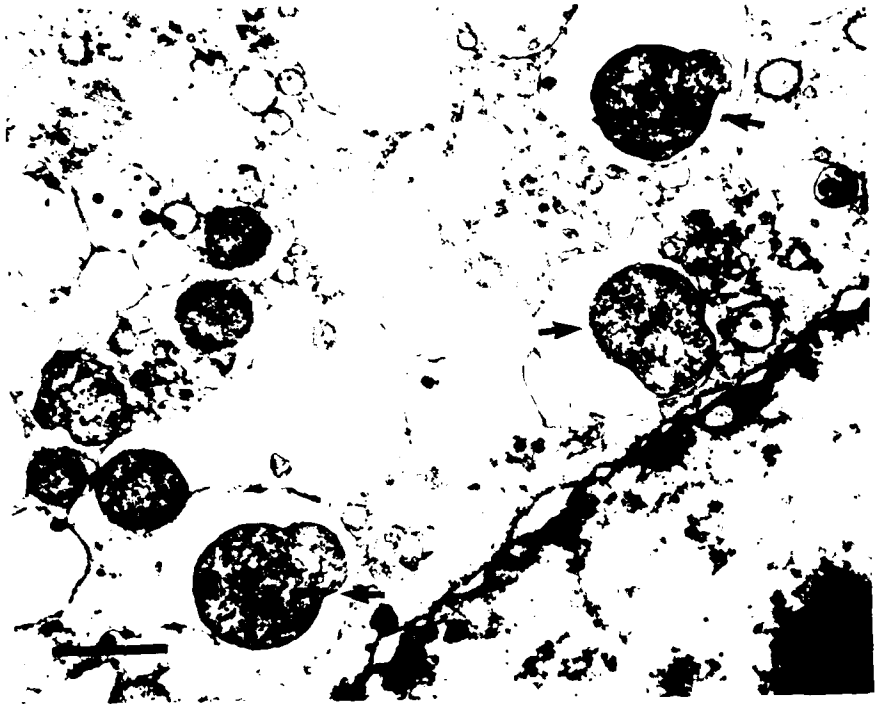

Figure 2. Transmission electromicrograph of reticulate bodies in McCoy cell at 10 hours post infection. Note three of the reticulate bodies are beginning to undergo binary fission. Bar = 1.0 um

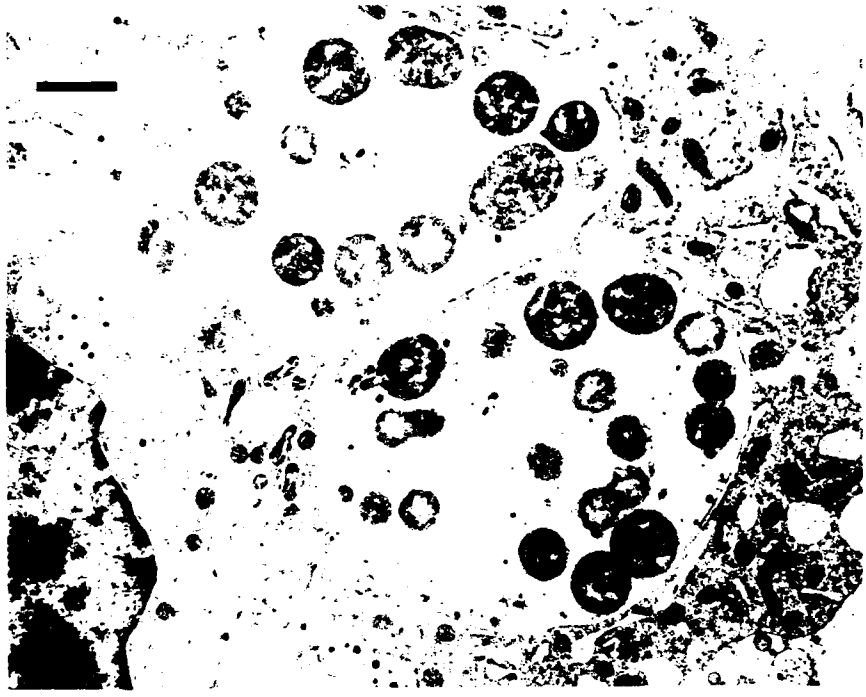

Figure 3. Two 24 hour old inclusion bodies of Chlamydia trachomatis within a McCoy cell. Note the predominance of reticulate bodies. Bar = 1.0 um

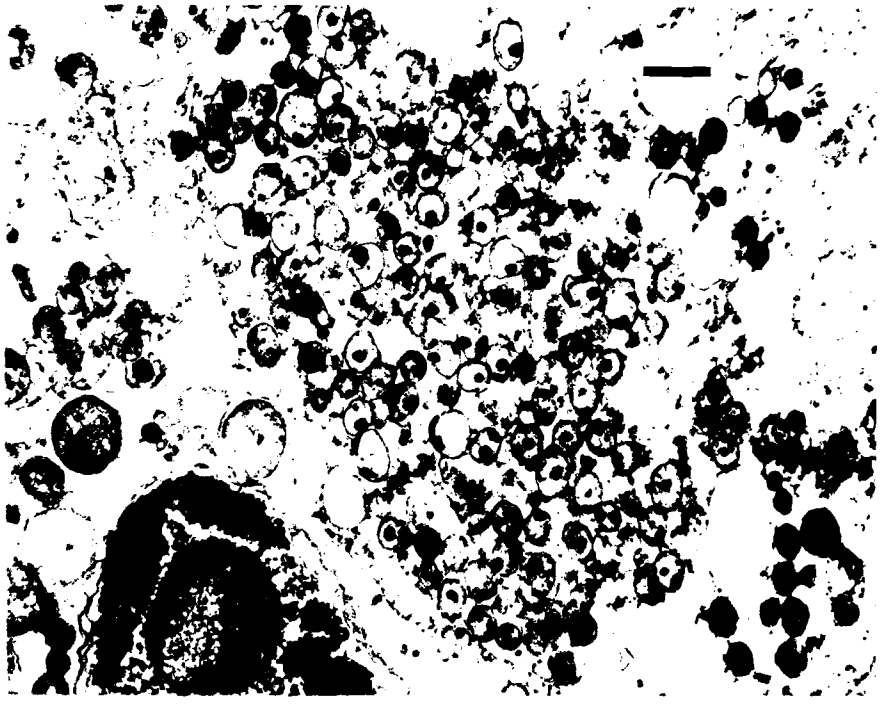

Figure 4. A 46 hour post infection inclusion body of Chlamydia trachomatis within a McCoy cell. Note the predominance of elementary bodies and intermediate bodies (reticulate bodies converting back to elementary bodies). Bar = 1.0 um

Antigens

Chlamydiae are antigenically complex organisms. They possess surface antigens that allow subdivision of the chlamydiae into genus, species, and serovar-specific groups (3,76).

Genus (Group) - Specific Antigens

All members of the genus Chlamydia share a common, heat stable, complement-fixing antigen (3). A host infected with either C. trachomatis or C. psittaci usually will have an immunologic response to this group antigen. The antigen is present on both elementary bodies and reticulate bodies (76). It has been compared with the lipopolysaccharide (LPS) of gram-negative bacteria and its biochemical composition is similar to LPS, although muramic acid and the peptidoglycan layer seen in free living gram-negative organisms are not present in Chlamydia (3). Examining the group antigen in greater detail, Brade et al. (10) reported that chlamydial LPS possesses two distinct antigenic determinants, one that is Chlamydia specific, and another which is responsible for cross-reactivity with enterobacterial-RE-type LPS. In contrast, Caldwell et al. (13) showed that the LPS of Chlamydia consists of at least three antigen domains; two are shared by the LPS of certain gram-negative organisms and one is unique to chlamydial LPS. Another group antigen has been described which is common to both species of Chlamydia, but is different from the group

antigen described above because it is heat labile (3).

Species - Specific Antigens

Caldwell and co-workers found a heat-labile protein with a molecular weight of 155,000 daltons to which antisera was made that reacted to all 15 human serovars of C. trachomatis (12 TRIC and 3 LGV), but not to the mouse C. trachomatis biovar or C. psittaci (3,76). Salari and Ward isolated a polypeptide that is probably the same antigen and suggested that it may be located in the chlamydial outer membrane (3,76). Caldwell and Schachter demonstrated the presence of species-specific antigenic activity in the major outer membrane protein (MOMP) of C. trachomatis (3). The protein consists of up to 60% of the outer membrane and also contains serovar-specific and sub-species specific epitopes.

Type (Serovar) - Specific Antigens

The type specific antigens are common only to subgroups of strains within chlamydial species (3,76). Wang and Grayston (159), using microimmunofluorescence, showed that members of the species C. trachomatis could be classified into 15 serovars indicating the presence of serovar-specific epitopes on these organisms. There are confusing data (3,76) concerning the extraction and isolation of these antigens, but it does seem certain that the type-specific antigens are located in the major outer membrane protein (3).

Sub-species - Specific Antigens

Wang and Grayston showed cross-reactivity between the 15 serotypes of C. trachomatis when using the micro-immunofluorescence test (76). This cross-reactivity is attributed to sub-species specific antigens and has been used to classify the 15 serovars into two major complexes (76): (1) the B complex (B, Ba, D, E, L1, and L2) and (2) the C complex (C, J, H, I, and A.). Serovars G and F cross-react with both complexes, but to a greater extent with the B complex. Serovars K and L3 are just the opposite and cross-react to a greater extent with the C complex. As stated above, these antigens are thought to be located on the MOMP.

Human Immune Response to Chlamydiae

In primary chlamydial infections, the initial response is the collection of polymorphonuclear leukocytes at the infection site followed by the development of both humoral and cell mediated immune responses (135). The extent to which the human immune response can eradicate chlamydial infections or prevent reinfection is presently unclear. It is clear, however, that a single infection will not result in reliable immunity to reinfection (123). One indication, however, that the immune system may play a role in host defense is that the prevalence of infection in women decreases with age. The immune response may help to clear some acute infections and limit others, but in chronic infections it may contribute to tissue damage. It is also possible that relative degrees of immunity may exist which in some cases may prevent reinfection, but in other situations may be overcome by a sufficient inoculum of elementary bodies (123). Further studies are needed to identify which factors influence the immune system's ability to prevent, control, and eradicate chlamydial infections.

Chlamydial Infections

Chlamydia psittaci Infections

C. psittaci causes the human disease called psittacosis when contracted from psittacine birds such as parrots and called ornithosis when contracted from non-psittacine birds such as poultry (122). The human disease occurs in two forms, a pneumonic form and a systemic (toxemic) form without a respiratory component (92). In the United States (U.S.), 100-200 cases are reported annually (127). These are mainly occupational associated infections with main reservoirs being infected poultry and infected pet birds. Even though imported birds are treated with antibiotics upon export to the U.S. (122), serological and culture testing show these potential pets may still harbor the organisms (39). Transmission to man may be indirect by inhalation of infected dried avian excreta, or from direct contact with infected birds (77). However, many cases cannot be traced to contact with birds. Human to human transmission is considered rare, but may occur more readily than previously thought (39).

Mammalian, distinct from avian strains of C. psittaci, are common pathogens of lower mammals, causing a number of diseases which are economically important (122). Human infection with mammalian strains of C. psittaci appear, however, to be rare. Cases of human infection have been reported after laboratory exposure and, in a few cases, after exposure to infected mammals (122). In 1985 a case

was reported of an abortion due to C. psittaci infection in a pregnant women who had close exposure to aborting ewes (128).

Diagnosis of psittacosis requires either recovery of the organism by culture or by demonstration of an immune serological response (77). There is a marked overlap between features of different pneumonias making an early clinical diagnosis difficult. Tissue culture is seldom attempted due to the sporadic nature of the disease. Therefore, psittacosis is still predominantly diagnosed retrospectively by complement-fixing antibodies to the group antigen (77). Tetracyclines are the treatment of choice and use of these antibiotics has reduced the mortality rate of psittacosis from 20-40% to 1% (77).

TWAR Stain Infections

TWAR is an acronym that reflects the history of the first two isolates. TW is for Taiwan, the geographic site of the first isolate from the eye of a child, and AR for acute respiratory disease indicating the clinical condition associated with the second isolate (127). Grayston et al. (37) presented evidence that TWAR is an important respiratory pathogen in college students. Using serological testing and culture, the organism was linked to pneumonia, bronchitis, and pharyngitis. By using a micro-immunofluorescence serologic test, the TWAR organisms have been etiologically associated with pneumonia epidemics in teenagers and young adults throughout the Scandinavian

countries (86). Marrie et al. (86) tested for TWAR in adult patients with community-acquired pneumonia and concluded that the TWAR organism also causes pneumonia in older adults and in individuals with chronic disease who require hospitalization.

Pneumonia associated with TWAR infection is clinically similar to mycoplasma pneumonia (37). Pharyngitis often accompanied by laryngitis is a common presentation. The disease may range from mild pneumonia in young patients to severe or fatal pneumonia in hospitalized and older patients (127).

Currently it is very difficult to diagnose TWAR infections. Microimmunofluorescence testing using TWAR antigen is currently the most sensitive serological test, but is not available in most clinical laboratories (127). The genus-specific complement-fixation test may be useful, but is often difficult to interpret due to other cross-reacting chlamydial group antibodies. The TWAR strains are difficult to culture, growing poorly in egg and cell cultures (68). Further research is needed to find a test which will be both definite and applicable in clinical laboratories.

Infection from TWAR may represent 10-20% of atypical pneumonias which is roughly half the number of cases caused by Mycoplasma pneumoniae (127). Although tetracycline is the treatment of choice, it may be preferable to treat with erythromycin which is also effective against M. pneumoniae

and Legionella species. The short course of treatment given for M. pneumoniae is not, however, sufficient for TWAR; a longer 14 day course is more effective and would treat all three organisms (127).

This newly discovered organism may help explain some of the sporadic cases of pneumonia in adults thought to be caused by C. trachomatis. Schachter et al. (127) retested specimens from some earlier studies that were thought to be pneumonias caused by C. trachomatis and reported that many of these infections may, in fact, have been caused by TWAR strains. Further research is needed to determine the TWAR strains' true spectrum of disease.

Chlamydia trachomatis Infections

Chlamydia trachomatis is now recognized as the agent of the most common sexually transmitted disease in the U.S.. Over four million infections occur each year with an estimated direct and indirect cost of over 1.4 billion dollars per year (162). Three-fourths of the total cost is due to sequelae of untreated, often apparently asymptomatic infections (162). This emphasizes the need for an active screening program in high prevalence populations and a national system for surveillance of Chlamydia-associated diseases as recommended by the Centers for Disease Control (CDC) (12). Table 2 (8) lists the clinical spectrum of known C. trachomatis infections other than trachoma (which is rare in the U.S.).

Table 2. Clinical spectrum of *Chlamydia trachomatis* infections[a]

Infections[b]
Adults
Urethritis (≤ 50%)
Proctitis
Epididymitis (30%-50%)
Pharyngitis[c]
Lymphogranuloma venereum
Mucopurulent cervicitis (30%-50%)
Endometritis
Salpingitis (≤ 50%)
Perihepatitis
Infants[d]
Conjunctivitis (15%-30%)
Pneumonia (afebrile, approximately 30%)
Asymptomatic pharyngeal infection
Asymptomatic gastrointestinal infection
Otitis media[c]
Stillbirth[c]
Neonatal death[c]
Complications
Infertility
Endocervical dysplasia[c]
Ectopic pregnancy
Postpartum endometritis
Premature labor[c]
Rectal stricture
Reiter's syndrome[c]
Prostatitis[c]

[a]Adapted from: Bell, T.A., and J.T. Grayston. 1986. Center for Disease Control guidelines for prevention and control of *Chlamydia trachomatis* infections. Ann. Int. Med. 104:524-526.

[b]Numbers in parentheses show prevalence of *C. trachomatis* in associated disease (U.S. Population).

[c]A relationship has not been firmly established between *C. trachomatis* and the infection or complication.

[d]*C. trachomatis* also causes symptomatic nasopharyngeal and asymptomatic vaginal infection in infants.

Ocular Infections

There are two types of C. trachomatis ocular infections: (1) trachoma, the first infectious disease linked to C. trachomatis and (2) the condition for which the species is named, and inclusion conjunctivitis, an acute infection that occurs usually as a complication of genital tract infections.

Trachoma: Trachoma is a chronic follicular keratoconjunctivitis caused by C. trachomatis serovars A-C (21). It is the most prevalent eye disease in the world with the World Health Organization estimating that 500 million people suffer from the disease (21). Trachoma is considered the most preventable cause of blindness in the world (122). The disease is a major public health problem in non-industrial emerging nations, particularly in Africa and Asia. In the U.S., it is rare, but currently found in mild forms among American Indians, Samoans, Filipinos and sporadically in immigrants from Mexico or the Far East (122).

Trachoma is essentially a disease of families occurring in young children. Transmission generally results from eye-to-eye transmission (122). The disease normally occurs in both eyes. Initial infection is normally self-limiting, but in endemic areas reinfection continually occurs and are complicated by secondary bacterial infections which interfere with the healing process and help perpetuate inflammation for years (122). Conjunctival scar may

contract causing the eyelid to turn inward so that eyelashes abrade the cornea. Fifteen to 20 years after initial infection, permanent lesions from trachoma can lead to blindness. Diagnosis of endemic trachoma can normally be made by examination of the eyes (21). Atypical forms of the disease without scarring require differential diagnosis from other bacterial and viral causes (21). Laboratory diagnosis is not required in endemic trachoma, but can be useful in diagnosis of atypical trachoma and for epidemiological studies (21,122). Tetracycline, erythromycin, rifampicin, and sulfonamides are all effective in treating trachoma, although none of these is considered ideal treatment (122). There is no vaccine available today. Earlier vaccines produced some short-lived protection, but some vaccinated individuals developed more severe disease due to hypersensitivity (126).

Inclusion Conjunctivitis: C. trachomatis serovars D-K may cause inclusion conjunctivitis in adults and newborn infants (see below for discussion of necnatal conjunctivitis). Adults may transfer organisms by hand from genital discharge to the eye (143). In adults, it is an acute follicular conjunctivitis and normally follows a self-limited course. Sometimes, however, the organism persists and clinical features consistent with trachoma may develop, but blindness rarely occurs (33,83,143). Laboratory diagnosis includes isolation by culture, cytologic demonstration by Giemsa stain, or direct fluorescent

antibodies. Oral administration of either erythromycin or tetracycline is recommended for treatment (12).

Male Genital Tract Infections

C. trachomatis is the major cause of both non-gonococcal urethritis (NGU) and post-gonococcal urethritis (PGU) in young men. It has been estimated that in the U.S. 1.55 million diagnosed infections by C. trachomatis occur annually in men (162). C. trachomatis has been diagnosed in up to 50% of the men with cases of NGU and in approximately 15-30% of heterosexual men with gonococcal urethritis (12).

Clinically, Chlamydia-positive NGU cannot be differentiated from Chlamydia-negative NGU. Both have a 7-21 day incubation period with symptoms of dysuria and mild-to-moderate whitish or clear urethral discharge (143). Up to 30% of heterosexual men with NGU have few or no symptoms (12,60,162), however, many men with asymptomatic NGU will still exhibit four or more polymorphonuclear leukocytes (PMN's) per 1000X microscopic field on gram stain of urethral secretions (143).

In patients with simultaneous Neisseria gonorrhoeae and C. trachomatis genital infections, the infection is often diagnosed as gonorrhea and the patient is then treated with penicillin alone. In these cases, the penicillin does not eradicate the chlamydial infection, leading to PGU. To deal with this problem, the CDC (12) has in the past recommended an ampicillin and tetracycline regimen as the treatment of choice for uncomplicated gonococcal infection in adults.

Homosexual men have a lower prevalence rate of both chlamydial NGU and PGU than heterosexuals (12). However, homosexual men who practice rectal intercourse are frequently infected with C. trachomatis and may develop proctitis. These cases of proctitis are usually less severe than those caused by LGV serovars (see below), and are often asymptomatic (143).

Infected males, especially if asymptomatic, who are not treated serve as a reservoir to spread the disease and a small minority will go on to complications. Washington et al. estimated that 80,300 cases of chlamydial epididymitis occur each year in the U.S. (162). C. trachomatis has been cultured from 30-50% of male patients with acute epididymitis (12). The organism is the leading cause of epididymitis among men less than 35 years old (12,143). Serological evidence has pointed to C. trachomatis as a possible cause of non-acute "abacterial" prostatitis, but cultures for the organism have failed or positive specimens have been suspected to be due to urethral contamination. Poletti et al. (104) has provided firmer evidence by culturing transrectal aspiration biopsies of the prostate and isolating C. trachomatis in 10 of 30 patients. This specimen collection method has the advantages of including less prostatic fluid which is thought to have an inhibitory effect on the formation of chlamydial inclusions in tissue culture cells, and lessening the chance of chlamydial contamination from the urethra. Although these findings

suggest C. trachomatis may have an etiological role in non-acute abacterial prostatitis, further studies are required to confirm this association.

Reactive Arthritis and Reiter's Syndrome

Reactive arthritis (RA) refers to initiation of inflammatory joint and other musculoskeletal lesions by an infection at a site distant from the joint while the joint itself is sterile (at least by conventional methods) (61). RA represents one of the most common forms of inflammatory joint disease among young adults. In RA associated with genital infection, males predominate over females by 10 to 1 (61). Approximately one male per 100 with NGU will develop RA (62). There appears to be an association of the disease with the presence of the HLA B-27 antigen (61,62). RA is normally self-limiting, but may become chronic and destructive with extra-articular lesions. Approximately 1/3 of the patients with reactive arthritis also have Reiter's syndrome (62). Reiter's syndrome refers to the triad of arthritis, genital inflammation, and ocular inflammation (61). Characteristic skin and mucous membrane lesions may also be associated with the syndrome. Keat et al. (62), using direct fluorescent antibody staining, demonstrated elementary bodies in 5/8 joint specimens of patients with sexually acquired reactive arthritis while all control patients remained negative. They feel this may be the key to demonstrating the etiology of RA and that further investigation is necessary to determine if the organisms are

viable which would have broad implications in treatment.

Female Genital Tract Infections

Infections of the female genital tract are the major source of human morbidity and mortality due to Chlamydia. It is estimated that 2.01 million diagnosed infections due to C. trachomatis occur annually in women (162). Age, (young women), number of sex partners (multiple partners), socioeconomic status (poor), and race (non-white) are associated with increased risk of C. trachomatis infections (12). C. trachomatis is, however, more common than N. gonorrhoeae in middle class white women attending universities or females of similar socioeconomic background attending family planning clinics.

The most common manifestation of C. trachomatis in women is cervicitis. The organisms have been isolated in 30-50% of the women with mucopurulent cervicitis (12). Nineteen percent of the women show signs of hypertrophic ectopy of the cervical epithelium (an area of ectopy that is edematous, congested, and friable) (143). Up to 50% of women diagnosed with N. gonorrhoeae are co-infected with C. trachomatis, making it important to treat diagnosed gonorrhea patients for both organisms (12). As in men, chlamydiae can cause urethritis (urethral syndrome) in women (143). This syndrome has two symptoms, dysuria and frequency without the presence of a usual uropathogen. There is, however, no firm set of patient characteristics or clinical symptoms which can diagnose chlamydial genital

infections; up to 70% of infected women may be asymptomatic (12,162).

Asymptomatic women serve as a reservoir of infection, but more importantly, are at significant risk for developing complications. Complications from untreated chlamydial infections include: pelvic inflammatory disease (PID) which may lead to more severe consequences such as ectopic pregnancy, infertility, or death from a ruptured ectopic pregnancy. C. trachomatis is responsible for 30-50% of the 400,000 cases of PID diagnosed each year in the U.S. (162). It is also estimated that C. trachomatis is responsible for 8,000 infertility consultations, 14,000 ectopic pregnancies, and 280 deaths per year (162). Other complications include perihepatitis (Fitz-Hugh-Curtz syndrome) (82,143), endometritis (58,143), postpartum and post-abortive endometritis (5,143), and periappendicitis (82). C. trachomatis has also been implicated as a possible cause of endocervical dysplasia, but this remains to be proven (82,143).

It is now thought that the ascending spread of chlamydiae in the female genital tract occurs canalicularily, through the cervical channel, the endometrial cavity, and the fallopian tubes into the peritoneal cavity and to the surface of the liver (82). Risk factors for developing upper genital tract infection are presently unknown and further studies of ascending complications are required.

Adult Respiratory Infections

Chlamydia trachomatis had been implicated as a cause of acute pharyngitis, but some recent studies (30,52,96) of adults and adolescents indicated an infection rate of less than 1%. However, Jones et al. (59) recently reported isolating C. trachomatis from the pharynx in 3.7% of asymptomatic men and in 3.2% of asymptomatic women. In the women, but not the men, an association was shown with oral-genital sex. It appears, however, that C. trachomatis plays a very limited role, if any, as a cause of acute pharyngitis in adults.

It has also been suggested that C. trachomatis may cause pneumonia in normal adults as well as immunosuppressed adults (148). Serological studies have, however, played a major role in these diagnoses. Serologies of adults have been shown to be unreliable indicators of current infections so this evidence must be regarded with caution (79,130). Moncada et al. (89) reported that C. trachomatis does not appear to be an important respiratory pathogen among acquired immune deficiency syndrome patients. As discussed earlier, the newly identified TWAR psittaci strain is an etiological agent of pneumonia in adults and may be responsible for many of the earlier serologically positive cases attributed to C. trachomatis (127). Further studies using tissue culture isolation are needed to demonstrate the role, if any, of C. trachomatis as an etiological agent in adult pneumonias.

Infections in Infants

Chlamydia trachomatis infection in infants is a complication of maternal genital tract infection generally acquired during vaginal delivery through an infected cervix. Chlamydial infection contracted through cesarean section is rare (45). Since most studies report chlamydial cervical infections in approximately 8-12% of pregnant women, it is not surprising that Chlamydia is the most common cause of neonatal eye infections and of afebrile interstitial pneumonia in infants less than six months old (12). It has been estimated that C. trachomatis is responsible for approximately 73,800 cases of conjunctivitis and 39,100 cases of pneumonia each year (162). Serological evidence indicates that approximately 70% of infants born to infected mothers are infected prior to six months of age (45).

Neonatal conjunctivitis is the most common manifestation of C. trachomatis infection in newborns. About 20-25% of infants born to infected mothers are brought to a physician for conjunctivitis (45). Chlamydial conjunctivitis is usually recognized from five to 14 days after delivery (72). Infections range from asymptomatic to severe purulent conjunctivitis. The prophylactic silver nitrate eyedrops that newborns receive for prevention of gonococcal ophthalmia do not prevent chlamydial ophthalmia (45). Prophylactic erythromycin or tetracycline ointment have been investigated as control measures, however, both have potential problems and further research is needed

(45,140). For treatment of diagnosed chlamydial conjunctivitis, oral therapy with these agents is recommended and has the advantage of also clearing potential nasopharyngeal infections and decreasing the further risk of pneumonia.

Beem et al. (7) characterized the syndrome of chlamydial pneumonitis and/or bronchiolitis. The risk of pneumonia or bronchiolitis from an infected mother symptomatic enough to be brought to a physician's attention is approximately 5-10% (45). Chlamydial pneumonia has a later onset than conjunctivitis and usually occurs between three to 16 weeks of age (72). Approximately 50% of infants with chlamydial pneumonia will have had conjunctivitis. Normally infants with chlamydial pneumonia are afebrile, do not appear to be ill, but have a dry, staccato cough. Wheezing may also be present with physical findings of diffuse rales, tachypnea, and, occasionally, otitis media (72). Erythromycin or sulfisoxazole are the drugs of choice for C. trachomatis infant pneumonia (72,140).

C. trachomatis can also be recovered from the pharynx and gastrointestinal tract of asymptomatic neonates born to infected mothers (8,45). The organism has also been associated with early acute otitis media, possible chronic lung disease due to earlier chlamydial pneumonia, and gastroenteritis (45). Some studies suggest that the organism is associated with premature birth, low birth weight, stillbirth, and neonatal death (8,45). Further

studies may define the actual role, if any, that chlamydiae play in these conditions.

Lymphogranuloma Venereum

Lymphogranuloma venereum (LGV) is an invasive rather than localized venereal disease caused by C. trachomatis (LGV biovar) serovars L1-L3. LGV is found worldwide, but is more common in tropical countries (134). The disease is uncommon in the U.S. with most cases occurring in travelers returning from endemic areas (121). The clinical course of LGV is divided into three clinical stages (134): (1) initial infection and early lesions (papule, shallow ulcer, or herpetiform lesions) may occur in the male or the female genital sites, but may also be found in extragenital sites; (2) the secondary stage involves swelling and inflammation of regional lymph nodes; often the inguinal nodes in males and retroperitoneal nodes in females. Males may present with painful enlarged buboes; (3) the tertiary stage or genito-anorectal syndrome includes many different conditions. It usually results from progressive spread of the disease with hypertrophic and necrotic lesions. This condition is more common in women where it may involve the lower intestinal tract with such conditions as esthiomene (Greek: meaning eating away), rectovaginal fistulae, proctitis, and rectal strictures. Normally men with LGV proctitis can be distinguished from men with other forms of C. trachomatis proctitis by the severity of disease (134). LGV is a systemic disease and a number of other

complications may occur (134). Laboratory diagnosis originally used the Frei test which was a skin test for a delayed hypersensitivity reaction. This test is insensitive and no longer used (121,134). The genus-specific complement-fixation test may help support the diagnosis. The test must be carefully interpreted, but is still used because normally LGV titers of infection are higher than non-LGV titers of infection stimulated by cross-reacting trachoma serovars (121,134). Isolation of LGV by tissue culture is the definitive diagnostic test. The bubo pus or biopsy tissue must be diluted to prevent toxicity to cells (134). The optimal regimen for treatment of LGV is still undetermined and the response to therapy is variable. Tetracycline or sulfonamides are normally used to treat LGV (121,140).

Antimicrobial Susceptibilities

There is no set reference procedure to test for C. trachomatis antimicrobial susceptibility. The simplest procedure is to infect a cell culture with a standard inoculum and then to add growth medium with different concentrations of the antibiotic being tested. The minimum inhibitory concentration is defined as the antibiotic concentration which inhibits 50-100% of the inclusion forming units (123). A more accurate test, that is less likely to overestimate the activity of an antibiotic, is the minimum bacteriocidal concentration. This level is defined as the first antibiotic concentration in a culture that shows no inclusions after one passage (123).

A number of antibiotics have been reported with consistently high in vitro activity against C. trachomatis: the tetracyclines (doxycycline, minocycline, and tetracycline hydrochloride); the macrolides (erythromycin, rosaramicin, spiramycin; rifampicin (although resistance may emerge); and the sulfonamides (140). Antibiotics with little or no apparent in vitro activity against C. trachomatis include the aminoglycosides, the aminocyclitols (spectinomycin), nalidixic acid, trimethoprim, vancomycin, metronidazole, lincomycin, cephalosporins and antifungal agents such as nystatin and amphotericin B. This is why gentamicin, streptomycin, vancomycin and antifungals may be used in transportation and growth media to prevent bacterial and fungal contamination in Chlamydia cultures (140). Most

penicillins exhibit intermediate activity against chlamydiae and require very high concentrations in vivo to eradicate the organisms (123,140). If penicillin is removed from the organisms, the life cycle may continue. As discussed above (in "Antigens"), the chlamydial cell wall is similar in composition to gram-negative organisms but lacks muramic acid, therefore, penicillins mode of action against chlamydiae must be different than for other bacterial organisms (123). Clindamycin and chloramphenicol also exhibit intermediate activity against chlamydiae (140).

Tetracycline or doxycycline is usually the drug of choice (12,140). Erythromycin or sulfonamides are also often used (pregnant women are normally prescribed erythromycin). Treatment for chlamydial infection normally requires antibiotics for at least seven days (12,140). When taken as prescribed, tetracycline and erythromycin are highly effective with a greater than 95% cure rate. No tetracycline-resistant chlamydiae have been described (12). Because many individuals diagnosed with N. gonorrhoeae are also co-infected with C. trachomatis, the CDC recommends treating these individuals with both a beta lactam and tetracycline. Washington et al. reported that dual treatment of penicillin-susceptible gonococcal cervical infections with ampicillin and tetracycline to be cost-effective (161).

Due to the unavailability of diagnostic testing, physicians usually treat nongonococcal NGU and mucopurulent

cervicitis infections empirically. When using empiric treatment there is, however, the problem of antibiotic resistance developing in other microorganisms (144). Also, antibiotics in these empirical regimens must be effective against other possible etiologic agents as well as Chlamydia (140). Tetracyclines are effective against most strains of Ureaplasma urealyticum, and Mycoplasma hominis, although increasing resistance by these organisms is being seen (140).

CDC recommends different regimens with different time tables based on the type of infection or complication diagnosed (12). To control chlamydial infections, it is imperative to check for regimen compliance by follow-up visits, and to treat contacts, thus preventing reinfection and further spread of disease.

Control of Chlamydial Infections

The Centers for Disease Control (12) and other authors (2,42,43,144) have recommended measures to control chlamydial infections. Two major points are seen in many suggested control guidelines: (1) increased screening of at-risk patients (2,12,42,43,144) and (2) advocacy of a national policy for reporting C. trachomatis to state laboratories and the CDC (12,42,144).

Nettlemen et al. (97) evaluated the cost-effectiveness of culturing for C. trachomatis in a sexually transmitted disease clinic. They concluded that empiric treatment of all patients attending the clinic was the most cost-effective strategy, followed by empiric treatment only of women at high-risk and culture-based treatment of women at low-risk. They did not find culturing of men to be cost-effective. Phillips et al. (103) reported that using rapid nonculture testing in screening women is cost-effective if the prevalence rate is above 7%, and that culturing would be cost-effective if the prevalence rate is 14% or greater. Presently the major drawback to routine screening is the absence of a single test which can diagnose C. trachomatis with nearly 100% accuracy and this is why some investigators advocate empiric treatment in high prevalence populations. The availability of rapid nonculture tests makes the goal of screening high risk populations for C. trachomatis realistic but the serious problem of false positive test results must be recognized and dealt with explicitly.

Laboratory Diagnosis of Chlamydial Infections

Cytology

Microscopic examination of Giemsa-stained scrapings of conjunctival, cervical, and urethral epithelial cells for the presence of characteristic intra-cytoplasmic inclusions was the primary method of diagnosing chlamydial infection until 1957 (122). Presently it is not recommended because of poor sensitivity, except in cases of infant conjunctivitis where staining with Giemsa or monoclonal fluorescent antibodies has shown 95% sensitivity when compared with tissue culture. Papanicolaou smears have also been used to diagnose chlamydial infection, but are not recommended due to low sensitivity and specificity when compared with tissue culture (18,27,29,31,108,139,165).

Serology

Serum antibody assays are of limited value for the routine screening and diagnosis of chlamydial infections, and remain primarily a research tool (122,123). Due to the complexities in antigen preparation and test performance, as well as to the absence of diagnostic utility, most clinical laboratories do not perform chlamydial serologic studies. Presently the major limitation to serologic diagnosis of chlamydial infection is the high background of chlamydial antibodies present in sexually active populations, making it difficult to clearly relate present titers to recent or active infections in individual cases (47). Three types of

assays are currently used: (1) complement fixation (CF), (2) microimmunofluorescence (MIF), and (3) enzyme immunoabsorbent assay (ELISA).

Complement fixation: This test is based on the complement fixing genus-specific antigen (group antigen) and recognizes all Chlamydia. The test is useful in the diagnosis of the invasive syndromes of lymphogranuloma venereum and psittacosis (122,123). These diseases normally produce higner titers of antibodies than seen in ocular and genital infections of C. trachomatis, but diagnosis may be complicated due to (1) the lack of an acute specimen to show seroconversion (four-fold rise in titer) with a convalescent specimen and (2) interfering high background of cross-reactive non-LGV C. trachomatis group antibodies. The test is relatively insensitive and not useful for the diagnosis of other chlamydial infections including trachoma, inclusion conjunctivitis, or genital tract infection because high titers to the group antigen do not normally occur in these syndromes (47).

Microimmunofluorescence: The MIF test was introduced in 1970 by Wang and Grayston (159). It detects antibodies to the serovar-specific antigens of C. trachomatis. It was used initially to define the different serovars of trachoma-inclusion-conjunctivitis agents, and has been used extensively in sero-epidemiologic studies of both ocular and genital tract infections. The method is more sensitive than CF and can be used to measure IgM, IgG, or IgA antibodies

(47). However, due again to the high background of antibodies in sexually active populations and to cross-reaction between sub-species specific antibodies, an IgG response is difficult to interpret (123). IgM antibodies are more specific for current infection, but are short-lived and are found in only 28% of individuals with diagnosed active infections, a rate similar to that found in apparently non-infected individuals (122). For these reasons the MIF test is too insensitive to be useful in diagnosing adult chlamydial ocular or genital infections (122,123,130). Due to the high prevalence of chlamydial antibodies in sexually active populations, a negative MIF is, however, a specific indicator of non-infection with C. trachomatis (123). Schachter et al. (132) have shown that the MIF test using IgM may be the diagnostic test of choice in diagnosing chlamydial pneumonia in infants. However, because of the difficulty of the procedure, it is not readily available.

Enzyme-Linked Immunosorbent Assay (ELISA): Recently ELISA kits have become commercially available to test for chlamydial antibodies. The method is simpler to perform than MIF and is similar in sensitivity, but has similar drawbacks as a diagnostic test. It is not recommended for diagnosis of adult ocular and genital infections (47,135). However, Mahony et al. (79) recently reported an ELISA procedure which was 100% sensitive and specific in the diagnosis of 17 infants with chlamydial pneumonias; they

feel that ELISA may permit more laboratories to diagnose perinatal infections.

Tissue Culture Isolation

As mentioned above (in "Historical Background"), prior to 1965 isolation of Chlamydia was performed in chicken egg embryos. It was difficult, time consuming, and not a feasible diagnostic procedure for clinical laboratories. In 1965 Gordon and Quan (36) introduced a tissue culture technique using centrifugal force to promote the uptake of C. trachomatis by irradiated McCoy cells. This technique was four times more sensitive in isolation of Chlamydia than the yolk sac method (35). Tissue culture isolation made it possible for some clinical laboratories to begin routine diagnosis of ocular-genital infections caused by C. trachomatis. Since 1965 the tissue culture procedure has become easier and more sensitive, and although it is far from perfect, it is currently considered the reference method for diagnosis of C. trachomatis (125). There are many steps to the tissue culture procedure and these will be now be described in some detail.

Specimen Collection: The first step in culture for Chlamydia is to obtain an adequate specimen. Proper collection of a specimen involves many factors (84), including the use of a non-toxic swab, a proper collection method for the site being cultured, use of an appropriate transport medium, and adequate storage of the specimen prior to culturing.

The swab used for culture should be non-toxic to Chlamydia and the proper size for the site from which the sample is obtained. Mardh et al. (85) reported a larger number of inclusions were obtained with cotton-tipped aluminum swabs and rayon-tipped plastic swabs than with calcium alginate-tipped aluminum and cotton-tipped wooden swabs. From these findings, it appears calcium alginate fibers and wooden shafts may be toxic to Chlamydia. This was also reported by Mahony and Chernesky (78) and, in addition, they found two out of three dacron-tipped swabs with plastic shafts to be toxic. Variations in inclusion counts seen in swabs of the same type may be due to differences in how tightly the swab fibers are bound to the swab shaft (78). It has been reported that both calcium alginate and rayon swabs may bind chlamydial elementary bodies irreversibly (138). Therefore, it may be best to immediately agitate the specimen tube containing the collection swab and remove the swab prior to storage (78,85). Besides swabs, the cytobrush is now available for the collection of endocervical specimens from non-pregnant patients. The cytobrush may be more efficient than a swab in obtaining endocervical columnar epithelial cells (23).

Mardh et al. (84) have described the proper collection techniques of specimens from specific sites. No matter what the specimen site is, epithelial cells must be collected and not just discharge or mucous. It has also been shown that C. trachomatis can exist in the urethra of women who have

negative cervical cultures as well as in co-infections. Jones et al. (57) reported that culturing pooled specimens from both cervical and urethral sites had a higher chlamydial isolation rate than culturing single specimens from either site alone, but a lower rate than the combined isolation from both sites. Manuel et al. (80) reported similar results, noting that pooling specimens caused no apparent increase in toxicity to the tissue culture monolayers.

Specimen Transport and Storage: A sucrose phosphate buffer (2-SP) is the best transport holding medium for C. trachomatis (1,81,155). The 2-SP supplemented with 10% fetal calf serum preserves infectivity of chlamydiae better than non-supplemented 2-SP (1). Antimicrobials should be added to the 2-SP to control growth of bacterial and/or fungal contaminants from urogenital specimens. The antimicrobials most often used are gentamicin or streptomycin, vancomycin, and amphotericin B or nystatin. Using 2-SP with antimicrobials will keep over 99% of cell monolayers from being affected from non-specific cytopathogenic effects and/or bacterial or fungal overgrowth (81). The container in which the 2-SP is stored may also influence recovery; it has been reported that chlamydiae isolated from 2-SP stored in glass tubes yielded fewer inclusions than chlamydiae stored in plastic tubes (85).

Once a specimen is collected, it must be stored properly until processing. There are conflicting data

(1,78,84,155) for specimen storage, but a conservative recommendation is: if cultures are going to be processed within 24 hours, store them at 4°C, otherwise freeze at -70°C or below until ready to culture (78).

Specimen Processing: In clinical laboratories, the tissue culture isolation of urogenital C. trachomatis usually includes the following steps: (1) a pre-rinse of the cell monolayer with DEAE-Dextran (not used in all laboratories); (2) inoculation of cell monolayer; (3) temperature controlled centrifugation; (4) phosphate-buffered saline (PBS) rinse (performed in some laboratories); (5) incubation; (6) staining for intracytoplasmic inclusion bodies; and (7) passage of negative cultures (performed by some laboratories).

Presently most clinical laboratories use McCoy cells (mouse fibroblast cells) for the isolation of C. trachomatis (114). HeLa 229 cells (originated from a human cervical cancer) and BHK-21 cells (derived from baby hamster kidney cells) are used in some clinical laboratories, but are most often employed in research situations (114). In clinical laboratories tissue culture cells are usually propagated in either one-dram shell vials or in multi-well cell culture trays to facilitate testing large numbers of specimens. Yoder et al. (167) developed a microtiter method using 96 wells. According to these investigators, the procedure offered a number of advantages over shell vials, such as being less expensive, taking less time, requiring less

space, having a lower contamination rate, but still having approximately the same sensitivity as one dram vials. Schachter (125), however, has presented data that microtiter plates are only 60% as sensitive as shell vials in screening for asymptomatic cervical infections.

Unlike the more invasive pathogens, C. psittaci and the C. trachomatis serovars L1-L3, the genital C. trachomatis serovars D-K do not infect untreated tissue culture cells efficiently (92,114). For adequate sensitivity, the aid of centrifugation or pretreatment with a polycation such as DEAE-Dextran is required. DEAE-Dextran pretreatment of McCoy cells results in slight enhancement of C. trachomatis infection if the specimen is not centrifuged onto the monolayer. However, when centrifugation is used, the effect of DEAE-Dextran pretreatment is negligible (114). This negligible effect was also seen in preliminary studies performed for this study. Using a clinical isolate, different dilutions of inocula were added to pairs of cell monolayers, one of which was pretreated with DEAE-Dextran while the other was not pretreated. All sets of monolayers were centrifuged and a comparison was performed by counting the number of inclusions per coverslip at each dilution. There was no significant difference in the number of inclusions counted between the pretreated and non-pretreated cells. In contrast, the effect of DEAE-Dextran pretreatment of HeLa cells has been reported to be significant (114,118).

Inoculation of 2-SP transport medium normally should include rapid thawing of the specimen (if frozen) and agitation with sterile glass beads that break up cells to release infectious elementary bodies. Warford et al. (160) showed that sonicated specimens had greater than double the number of inclusions than non-sonicated specimens not agitated with glass beads in tissue culture. Further study is needed to compare agitating specimens with glass beads to sonicating specimens, and to determine whether combining the two techniques has an additive effect.

The optimal volume of 2-SP for inoculation of tissue cultures has not been determined. Most procedures suggest a range from 0.1 ml to 0.5 ml. In studies preliminary to those presented below, a clinical isolate at different dilutions was used to compare a 0.2 ml inoculum to a 0.5 ml inoculum by counting the number of inclusions per coverslip at each dilution. The 0.5 inoculum showed a significantly higher number of inclusions at all dilutions. Further evaluation with clinical samples is needed to determine whether the benefit of an increased sensitivity is greater than the possible detrimental effect of increased toxicity to the cell monolayers due to the larger inoculum size.

Centrifugation of the inoculum onto the cell monolayer is a major step that increases the infectivity of C. trachomatis in tissue culture. Theories of why there is increased infectivity include: that the centrifugal force precipitates elementary bodies onto the cell monolayer

overcoming repellent electrostatic forces, or that the centrifugal force changes the host cell membrane allowing increase uptake of elementary bodies (114). Most clinical laboratory procedures recommended centrifugation between 2000 and 3000 x G at 33-37°C for one hour. Reeve et al. (111) reported that centrifugation in excess of 3000 x G increases sensitivity only marginally. Darougar and co-workers (20), however, reported increased infectivity of cell culture with centrifugation speeds up to 15,000 x G. Temperature regulation during centrifugation is important in promoting attachment and endocytosis of elementary bodies into the host cell (114). Permitting temperatures higher than 37°C leads to a decline in infectivity (81).

After centrifugation, some laboratories wash the cell monolayer with PBS or growth media. The purpose of the washing step is to reduce toxicity and contamination of tissue cells by removing mucous, red blood cells, other debris, bacteria, and fungi (23). In a study preliminary to those presented below, a clinical isolate was used to compare the number of inclusions per coverslip at different dilutions of non-washed versus twice-washed monlayers. At all dilutions, the PBS washed monolayers had lower inclusion counts than the non-washed monolayers. To prevent such a decrease in the number of inclusions formed, a 1-2 hour incubation prior to washing or adding of growth media as recommended in some procedures (114,124) may be beneficial by allowing endocytosis of the elementary bodies that would

be otherwise washed away. Laboratories that perform washes need to determine whether the decrease in toxicity is great enough to overcome this apparent loss of sensitivity due to PBS washing.

To increase both the number and size of inclusion bodies in tissue culture, investigators have designed methods for placing the cell monolayer in a stationary growth phase despite a rich culture medium (see table 3). In such an environment the chlamydiae do not have to compete for nutrients (114). When using McCoy cells, these methods have included pretreatment of the cells with irradiation, or chemicals such as cytochalasin B or 5-iodo-2-deoxyuridine (IUDR) which produce cytostatic effects. Presently in most laboratories, cycloheximide is the antimetabolite of choice because it eliminates the need to pretreat tissue culture cells before inoculation with chlamydiae. Cycloheximide is conveniently included in the chlamydial growth media which is added to the tissue monolayers after centrifugation. Inoculated tissue cells are usually incubated in the growth media for 48-72 hours at 37^{o}C with or without carbon dioxide. Cycloheximide is a glutaramide antibiotic which inhibits deoxyribonucleic acid and protein synthesis in eukaryotic cells, but not in the prokaryotic Chlamydia (114). In most studies, significantly more cytoplasmic inclusions have been found in tissue culture cells treated with cycloheximide than have been found when irradiation or the other two chemicals listed above were used (25,69,115).

Table 3. Various techniques for the isolation of Chlamydia trachomatis in eukaryotic cells[a]

Investigators, year published	Cell type	Cell Treatment	Interval from start of treatment until inoculation
Gordon & Quan, 1965	McCoy	Irradiation	7 days
Kuo et al., 1972	HeLa 229	DEAE-dextran	30 minutes
Blyth & Taverne, 1974	BHK-21	-	-
Wentworth & Alexander, 1974	McCoy	IUDR	3 days
Sompolinsky & Richmond, 1974	McCoy	Cytochalasin B	3 days
Hobson et al., 1974	McCoy	-	-
Ripa & Mardh, 1977	McCoy	Cycloheximide	-

[a]Adapted from: Ripa, K.T. 1982. Biological principles of the culture of Chlamydia trachomatis in cell monolayers. Scand. J. Infect. Dis. 32:25-29.

Cell Monolayer Staining: The objective of using the tissue culture technique is to produce detectable intracytoplasmic inclusions. The method used to detect intracellular inclusions is thus an important element in the culture technique. In clinical laboratories, three staining methods have been used to detect chlamydial inclusions (23): (1) Giemsa stain at 48-72 hours (see figure 5), (2) iodine stain at 48-72 hours (see figure 6), and (3) fluorescent tagged monoclonal or polyclonal antibody (FA) staining at 24-72 hours (see figure 7).

Giemsa is a reliable, permanent stain that is very sensitive when examined by an experienced microscopist or with the aid of dark-field microscopy (23). It is, however, time consuming to read and can be misinterpreted by inexperienced microscopists. In most laboratories it has been supplemented by the more convenient iodine or FA staining techniques.

Iodine is the most commonly used stain because it is simple and inexpensive. However, iodine is also the least sensitive of the three methods. Iodine stains the glycogen matrix found in inclusions formed by all serovars of C. trachomatis. It will not detect strains of C. psittaci whose inclusions do not contain detectable glycogen (23,92). Glycogen production peaks at about 48 hours and then declines rapidly (23). Therefore, for best results, the monolayer should be stained between 48 and 72 hours.

Using fluorescent tagged monoclonal antibodies is the

Figure 5. Giemsa stained inclusion body of Chlamydia trachomatis in a McCoy cell. Original magnification 400X.

Figure 6. Icdine stained inclusion bodies of Chlamydia trachomatis in McCoy cells. Original magnification 400X.

Figure 7. Fluorescent monoclonal antibody (Syva) stained inclusion bodies of Chlamydia trachomatis in McCoy cells. Original magnification 400X.

most sensitive and specific staining method available (125, 145,147). Stamm and co-workers reported that improved sensitivity was most evident in specimens with low numbers of inclusions (145). Staining with FA may take place as early as 24 hours, but the inclusions appear smaller and are more difficult to distinguish, requiring an experienced microscopist. The major drawbacks of using FA staining are the reagent cost and the requirement of a fluorescent microscope.

All staining methods require careful examination of the cells for inclusions. Inclusion bodies must be distinguished from artifacts by color, morphology, and location of the inclusion.

Passage of Tissue Cultures: Many laboratories perform "passes" of negative primary cultures. For passage, two or more (rather than one) McCoy cell monolayers are inoculated initially. A "pass" is defined by the following technique: sterile glass beads are placed into one or more of the duplicate 48-72 hours old tissue culture(s) and are agitated to release elementary bodies (if present) from the cell monolayer, an aliquot is then inoculated onto one or more fresh monolayer(s), and the culture procedure is repeated. The purpose is to detect low titer infections which were missed by the primary culture. Jones et al. (57) report that increasing the number of passages to five results in a marked increase in isolation of C. trachomatis. Schachter and Martin (133) dispute the need of so many passes and

report that the recovery rate of C. trachomatis in a vial system (Jones and co-workers used microtiter plates) is not markedly increased after one passage. They recommend one blind pass of negative cultures, with this being more important in asymptomatic patients.

Culture Variation

Tissue culture is the reference standard for diagnosing C. trachomatis infections (12,125,129). The most sensitive culture method currently available uses cycloheximide-treated McCoy cells in shell vials and staining with fluorescent monoclonal antibodies (12). However, even when using an optimal tissue culture procedure, the sensitivity is below 100% (12,125). Schachter (125) reports the sensitivity of tissue culture from asymptomatic women using a single culture to be approximately 75% (in this longitudinal study only 75% of the untreated culture positive women were positive upon reculture after the first culture attempt).

A few investigators have performed tissue cultures on consecutive swabs obtained simultaneously from patients in an attempt to answer two questions: (1) whether there is a cumulative effect, that is, whether increasing the number of cultures increases the isolation rate of C. trachomatis and (2) whether swab order has a sequential effect, that is, whether a later swab is more sensitive than an early swab in detecting C. trachomatis.

Embil et al. (24) tested 260 women from a sexually

transmitted disease clinic using five consecutive cervical swabs. They reported that the fourth and fifth swabs were more sensitive (80-82%) than the first and second swabs (67-69%) and that using two swabs increased the number of detectable cases by as much as 44.7%. They speculated that the later swabs had more cervical cells and recommended cleaning of mucous from the cervical os before obtaining culture specimens to reduce toxic substances which can disrupt cell monolayers. In contrast to the above study, Munday et al. (94) tested 104 women from a sexually transmitted disease clinic using three sequential cervical swabs. They reported that processing three swabs instead of one increased the isolation rate by only 2% and that later swabs did not result in the production of more inclusion bodies than the first swab. They concluded that using a single swab is sufficient for isolation of C. trachomatis and will contribute only slightly to the culture failure rate. Dunlop et al. (22) isolated C. trachomatis from 110 women using triplicate cervical swabs. The first swab yielded 59%, the second an additional 14%, and the third an additional 8%. They concluded that the use of a single swab underestimates the prevalence of chlamydial infections. This cumulative effect was also seen in infected women who had triplicate swabs cultured from urethral and rectal specimens. Singal et al. (136) tested 136 men with urethritis in a sexually transmitted disease clinic by culturing two consecutive swabs for the isolation of C.

trachomatis. However, swab 1 was tested for GC and C. trachomatis while swab 2 was tested for only C. trachomatis. The first swab isolated 74% and the second swab isolated 97% of the positive cases of C. trachomatis. The second swab had a higher inclusion count more frequently than the first swab. These investigators concluded that a second swab will improve rates of recovery of C. trachomatis. Hernandez et al. (46) tested three consecutive cervical swabs from 70 asymptomatic adolescents and four cervical swabs from 80 women in a sexually transmitted disease clinic. Both sets of patients were assayed using Chlamydiazyme (described below). Neither population showed a sequential effect, that is, earlier swabs tended to be positive more often in both groups.

The issues of whether there is a significant cumulative and/or a sequential effect from performing multiple cultures are not clear. As seen, conflicting published data on these questions exist. Further investigations using tne most sensitive tissue culture methods available, like the "Culture Variation Study" reported below, are required to address these issues.

Direct Non-Culture Rapid Diagnostic Tests

In the last four years, major advances have taken place in the diagnosis of infections due to C. trachomatis. Two types of antigen detection tests used to detect C. trachomatis in clinical specimens have been introduced: (1) direct fluorescent antibody (DFA) stains and (2) ELISA.

Also, it appears that a third rapid nonculture method will soon be available; the first FDA approved isotopic and non-isotopic nucleic acid probes for detection of C. trachomatis should be released in 1988.

Direct Fluorescence Antibody: In 1984 Tam et al. (149) reported the use of a fluorescein-conjugated monoclonal antibody to species-specific antigen on direct smears prepared from urethral or cervical secretions that demonstrated a sensitivity of 93% and specificity of 96% versus tissue culture. Since then, several manufacturers have produced fluorescent labeled antibodies to species-specific or genus-specific chlamydial antigens. Only the MicroTrak^{TM} kit (Syva, Palo Alto, CA) has been extensively evaluated in publications. The test utilizes monoclonal antibodies prepared against the major outer membrane protein present on all 15 known human serovars of C. trachomatis (the species-specific antigen) and does not cross-react with C. psittaci. The antibodies are labeled with fluorescein isothiocyanate and will attach to both elementary bodies and reticulate bodies. The total processing time is approximately 40 minutes and includes slide preparation, fixing, staining, washing, and examining for elementary bodies under a fluorescent microscope. From urogenital specimens, investigators report sensitivities ranging from 59.6% to 100% and specificities ranging from 83% to 99% when compared to tissue culture (6,16,19,26,32,48,55,70,71,74, 106,109,137,142,146,152,153,157,164). The test has been

reported as extremely sensitive and specific in diagnosing neonatal ophthalmia neonatorum (75). It is imperative, when comparing studies, to look at: (1) the tissue culture method used for isolation, (i.e. if using an insensitive tissue culture system, one would expect to see a higher sensitivity and a lower specificity of the DFA test); (2) the number of elementary bodies an investigator considers as positive, (i.e. the manufacturer recommends that 10 or more elementary bodies be seen for a positive result, therefore, an investigator using one elementary body as a cut-off would have different comparison results from someone using the manufacturer's 10 elementary bodies cut-off); (3) the specimen site being examined (i.e. female endocervix versus male urethra); and (4) the prevalence of C. trachomatis and other attributes of the population being studied, (i.e. the manufacturer warns that in low prevalence populations (5% or less) a positive result must be interpreted with caution, admitting lower specificity in low prevalence populations; also, the sex of population must be taken into consideration and whether the population was composed of asymptomatic, symptomatic, or both type of patients, since asymptomatic patients may have lower titer infections and therefore a lower sensitivity when compared to tissue culture).

Personnel reading the DFA slides must be able to distinguish true positive specimens from non-specific fluorescence based on color and morphology. Krech et al. (67) reported false positive fluorescence of Staphylococcus

aureus based on non-specific uptake of antibody by Fc receptors on protein A. Stamm (141) has reported binding of the antibody not only to S. aureus, but also to Peptostreptococcus productis and strains of N. gonorrhoeae. Harper et al. (44) have also reported various forms of non-specific fluorescence which could be misinterpreted by an inexperienced microscopist.

Advantages of using DFA are: (1) the microscopist can determine specimen-adequacy based on the presence of columnar epithelial cells, (2) uncomplicated transport and storage, (3) rapid processing time, and (4) in most cases it is less expensive than culture. Disadvantages include: (1) need for a high-quality fluorescent microscope, (2) subjective interpretation requires an experienced microscopist to distinguish specific from non-specific fluorescence, (3) although it may be cheaper than tissue culture, reagents are still expensive, (4) excessive eye strain from reading many specimens which makes this test less practical in high volume laboratories, and (5) low predictive value of positive results in low prevalence populations.

According to the manufacturer, the test kit is designed for urogenital and conjunctival specimens as well as rectal specimens from symptomatic patients. However, a number of investigators have used the test to diagnose chlamydial infections in other sites including: joint aspirates (62), nasopharyngeal secretions (9,98), endometrium (65), fallopian tubes (65), cul-de-sac (65), and epididymis (64).

The test may also be useful as a test of cure after antimicrobial therapy (71,95). Further studies are required to verify DFA's performance to diagnose C. trachomatis infections in these non-FDA approved specimen sites.

Enzyme-Linked Immunosorbent Assays: Presently a number of ELISA test are on the market or are being evaluated, but only one method, the Chlamydiazyme (Abbott Laboratory, N. Chicago, IL) has been extensively evaluated in publications. One other test, the IDEIA Chlamydia test (Boots-Celltech Diagnostic, Inc) has limited evaluation information available (15,28,38,87,107,156,).

In the Chlamydiazyme procedure (23), patient specimens are incubated with manufacturer-treated beads in microtiter plates. If a specimen contains chlamydiae, the chlamydial antigen adsorbs non-specifically (not a specific antibody-antigen reaction) onto the bead. After this incubation, a wash is performed followed by the addition of a polyclonal rabbit antibody to the genus-specific antigen (LPS). A second wash is then performed. Next an enzyme-conjugated polyclonal goat anti-rabbit antibody is added which reacts with the antigen-antibody complex if present on the bead. The beads are washed and a chromogen is added which will react with the enzyme-conjugate complex developing a color endpoint proportional to the amount of chlamydial antigen present. The optical density of each specimen is read using a spectrophotometer. The test is practically suited for laboratories which run large batches and can be completed in

approximately 5-6 hours.

Investigators report sensitivities in urogenital specimens ranging from 60.0% to 100% and specificities ranging from 85.6% to 98.1% when compared to tissue culture (4,6,16,40,48,51,56,71,73,88,90,93,102,106,112,117,137). As with DFA, when comparing studies, it is important to consider the tissue culture method used for isolation, the specimen site being examined, and the prevalence of C. trachomatis and other attributes (sex, asymptomatic or symptomatic presentation) of the population being studied. The type of test kit used (the original or the "improved" modified Chlamydiazyme procedure that became available in 1985) must also be considered when comparing studies (48).

In simultaneous studies of both Chlamydiazyme and DFA (Microtrak^{TM}) compared to tissue culture, the two methods are similar in sensitivity and specificity (6,16,48,71,106,137) (see table 4). Taylor-Robinson et al. (151) evaluated Chlamydiazyme using Microtrak^{TM} as the standard (because they felt that "it would provide a more stringent test") and showed a sensitivity of 58% for men and 67% among women with specificities of 99% and 89%. Unlike most investigators, they recommended the Chlamydiazyme not be used for testing clinical specimens.

According to the manufacturer, the Chlamydiazyme kit is designed for urogenital and conjunctival specimens. Hammerschlag et al. (41) evaluated the Chlamydiazyme versus tissue culture for the diagnosis of chlamydial

Table 4. Comparison of Chlamydiazyme and Microtrak with culture for detection of *Chlamydia trachomatis*

				Chlamydiazyme		Microtrak	
Reference	Clinic	Sex	Prevalence	Sensitivity	Specificity	Sensitivity	Specificity
6	Obstetrics	F	21.2%	96.3%	92.9%	98.1%	95.4%
16	Multiple	F	15.8%	98.3%	97.5%	87.9%	98.4%
		M	26.5%	70.0%	95.8%	70.0%	97.2%
48	Multiple	F	12.6%	83%	98%	73%	99%
		M	20.3%	83%	93%	78%	99%
71	Community Health	F	5.2%	78.4%	96.8%	81.1%	97.9%
106	Private Practice	M/F	8.1%	91.2%	96.9%	91.2%	97.9%
137	Obstetrics	F[a]	12.1%	85.7%	95.6%	84.6%	96.6%
	Gynecology	F[b]	9.1%	60.0%	95.9%	65.5%	97.2%

[a]Pregnant females
[b]Non-pregnant females

conjunctivitis and respiratory infection in infants. They reported sensitivities and specificities of 98% and 95% in conjunctival specimens, 87% and 92% from nasopharyngeal specimens in infants with conjunctivitis, and 100% and 100% for nasopharyngeal specimens from infants with suspected chlamydial pneumonia. Further larger studies are required to determine ELISA's ability in diagnosing C. trachomatis from respiratory specimens.

Testing sites not recommended by the manufacturer also require further study. It must be recognized when evaluating false positive ELISA's that some may be due to cross-reaction with certain gram-negative organisms. Saikku et al. (119) reported Chlamydiazyme tests detected five different strains of Acintobacter calcoaceticus subsp. anitratus at concentrations varying from 10^3 to 10^5 organisms per milliliter. Taylor-Robinson et al. (151) reported that the Chlamydiazyme antibody reacted with strains of A. calcoaceticus (10^5), Escherichia coli (10^7), Gardnerella vaginalis (10^6-10^7), Neisseria gonorrhoeae (10^4,$2x10^7$), and group B streptococci (10^7).

The IDEIA Chlamydia test uses a monoclonal antibody to the genus-specific lipopolysaccharide antigen of C. trachomatis for antigen capture (23). The same antibody with an enzyme conjugate is used to bind the captured antigen (if present). An enzyme substrate is then added. The product from the enzyme-substrate reaction participates in a second enzyme reaction when an amplifier is added,

producing a color change that is proportional to the quantity of chlamydial antigen that initially bound in the well (107). When compared to tissue culture, the test has shown sensitivities ranging from 67.6% to 99.9% (15,107,156). Concerning the evaluation that gave a 67.6% sensitivity, the manufacturer published a response claiming that the authors used an improper cut-off (28). Two reports (38,87) compared Chlamydiazyme directly to IDEIA on patients without use of a tissue culture standard. Both reported no statistical difference between the two procedures. However, one evaluation (38) reported dilution experiments indicating Chlamydiazyme had greater sensitivity than IDEIA. Further studies are required comparing Chlamydiazyme and IDEIA to tissue culture. As with the Chlamydiazyme, false positive results have been reported with this ELISA method (113,116).

The ELISA test may be the best screening method for large volume laboratories which do not have the capability to perform tissue cultures. The procedure has the advantages of: (1) rapid turnaround time; (2) in most cases its less expensive than culture; (3) specimens are easy to transport and store (specimens are stable refrigerated up to five days); and (4) interpretation of result is not subjective. Some disadvantages include: (1) the test must be run in large batches to be cost effective; (2) specimen adequacy cannot be checked; (3) as with DFA, ELISA specificity may be a problem in low prevalence populations; and (4) the assay reagents remain expensive.

Nucleic Acid Probes: The use of diagnostic nucleic acid probes to detect C. trachomatis is just beginning. Because two types of nucleic acid are present in most cells, the target sequence can be either DNA or RNA (66). DNA:DNA, RNA:RNA, and DNA:RNA double strand molecules can be formed by hybridization under appropriate conditions. DNA probes with broad or narrow specificity can be developed by selecting an appropriate nucleic acid sequence as the target.

Palva et al. (100) developed a sandwich hybridization assay using cloned chlamydial DNA as the reagent and demonstrated that nucleic acid hybridization assays could detect C. trachomatis in clinical specimens. The test required two separate nucleic acid reagents derived from two non-overlapping but adjacent regions of the chlamydial genome. One fragment was immobilized on a nitrocellulose filter in a single-stranded form and the other fragment was radio-labeled and used as a probe. Of 16 urogenital specimens tested, eight were positive by tissue culture. The DNA probe agreed with all 16 tissue culture results. Hyypia et al. (54) cloned a plasmid from an L2 serovar. All 15 serovars of C. trachomatis contained DNA sequences that hybridized with the ^{32}P labeled L-2 probe. Also, serovars L1, L2, and L3 showed significant homology with a probe using total DNA from L2 elementary bodies in southern blot and spot hybridization analysis. In further evaluations, Hyypia et al. (53) used nucleic acid spot hybridization to

detect chlamydial DNA in clinical specimens. Whole L1 chromosomal DNA probe labeled with ^{32}P detected chlamydial DNA not only in 31 out of 38 culture positive specimens but also in eight out of 30 culture negative specimens. To increase sensitivity and specificity, the authors concluded that either more sensitive probe systems or better sampling methods would be required.

In 1980 Lovett et al. had reported that C. trachomatis contained a 7-kilobase cryptic plasmid (50) (the same plasmid later used by Hyypia). In 1985 Palmer and Falkow cloned the plamsid into Escherichia coli and called it pCHL2 (99). They determined that the plasmid occurs as 10 copies per chlamydial chromosomal equivalent. Their study demonstrated the plasmid in all 15 human serovars of C. trachomatis and showed that the plasmid is closely related to, but different from, a plasmid of similar size isolated from C. psittaci. Horn et al. (49,50) used this plasmid tagged with ^{35}S-ATP to test tissue culture and cervical secretions prepared for cytology screening by in situ DNA hybridization. In a preliminary study, the probe detected as positive all 44 infected but one of 13 uninfected cultures (50). The probe was also used to test cervical smears and was 91% sensitive and 83% specific. The procedure entailed preparing cells for hybridization, hybridization, washing, autoradiography, and staining with hematoxylin. Positive specimens could be detected microscopically using 100X magnification by identifying

clusters or foci of developed silver grains overlying the cells. In further studies, DNA hybridization detected all 35 C. trachomatis infected tissue cultures and was negative for all 27 uninfected tissue cultures (49). The probe could effectively detect C. trachomatis in microtiter plates of 10 IFU/ml (inclusion forming units). Probe testing of cervical smears versus culture of 46 specimens, with 22 positive by culture, showed the procedure was 91% sensitive and 80% specific (49).

Pollice and Yang (105) used the pCHL2 plasmid non-isotopically labeled with biotin for in situ hybridization detection of chlamydial infections. They tested 13 serotypes of C. trachomatis between 15-24 hours after inoculation onto McCoy cell monolayers and were able to detect chlamydial infection in all cases. Their results were similar to those reported by Palmer and Falkow (105) using the same probe with a ^{32}P label. This method offered the advantages of not working with radioactivity and increased stability (two year) of the probe label. This biotinylated DNA probe is currently marketed by Enzo Biochem, Inc. for use as a research tool for the detection of C. trachomatis in cell culture or cell scrapings. The specimen is fixed to a glass microscope slide, biotin labeled probe is added, the DNA strands are denatured by heating, followed by a room temperature incubation to allow for hybridization. The slide is then washed to remove unbound probe, and an avidin-horseradish peroxidase

detection complex is added which binds to the biotin attached to the DNA probe. Hydrogen peroxide and aminoethylcarbozol are then added to react with the horseradish peroxidase yielding a red precipitate. The procedure leaves the cells intact and the red inclusions can be viewed using a light microscope (see figure 8). The manufacturer claims the probe can detect C. trachomatis in infected McCoy cells after only 22 hours of culture (34). Currently there is no information on the sensitivity or specificity of the Enzo Pathogene II^{TM} kit for the detection of C. trachomatis. However, in studies preliminary to those presented below, different dilutions of C. trachomatis G serovar were inoculated in triplicate onto McCoy cell monolayers. After 48 hours one monolayer was tested with the Syva culture confirmation FA, one with iodine, and the third with biotinylated DNA probe. The DNA probe showed fewer inclusions than either FA or iodine and was the least sensitive of the three methods.

In a clinical study, Pao et al. (101) reported that radioactive labeled DNA probes to C. trachomatis gave a higher sensitivity and specificity (91.7% and 95.3%) than Chlamydiazyme (68.8% and 94.7%) versus tissue culture. However, the DNA probes were radioactively labeled, required a nitrocellulose membrane filter sheet, and autoradiography for detection of hybrids. This method is labor intensive and time consuming, and is not feasible for use in a clinical laboratory setting.

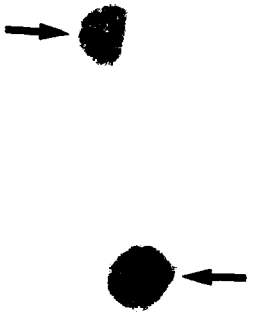

Figure 8. Biotinylated DNA probe (Enzo Biochem, Inc.) detection of inclusion bodies of Chlamydia trachomatis (LGV 2) in McCoy cells. Original magnification 400X.

Gen-Probe, Inc. (San Diego, CA) has the first two FDA approved DNA probe-based assays designed to detect C. trachomatis in direct urogenital clinical specimens. In one DNA probe assay, the probe is radioactively labeled with ^{125}I. The other DNA probe assay is called the PACE^{TM} assay system and the probe is non-isotopically labeled with chemiluminescent acridinium ester. The manufacturer claims the DNA probes are designed to detect all 15 serovars of C. trachomatis, do not react with C. psittaci, and do not react with other urogenital microorganisms.

Both probe assays differ from the conventional DNA probes used to detect Chlamydia described above because the Gen-Probe DNA probes are designed to bind to target chlamydial ribosomal RNA (rRNA) and use an in-solution hybridization method. Targeting rRNA has advantages because it is already single-stranded, has a greater number of copies for hybridization, and its sequences are considered to be highly conserved during evolution (66). An in-solution hybridization method requires no immobilization or nucleic acid purification and is much faster, allowing for rapid testing directly on clinical samples (66).

The procedure includes placing properly collected urogenital swabs into a lysing reagent to release rRNA, hybridization of DNA probe and sample in a 60^{o}C water bath, separation of hybridized probe by magnetic spheres which non-specifically bind the rRNA, washing to remove unbound DNA probe, and detection. In the isotopic assay, detection

of ^{125}I hybrids is measured in a gamma counter. For the non-isotopic assay, detection of acridinium ester hybrid is performed in a luminometer. When diluted alkaline hydrogen peroxide is added to an acridinium ester, a chemical chemiluminescent reaction releases light (163). The light is measured by a photomultiplier tube in the luminometer. Both DNA probe assays can run 40 specimens in approximately three hours.

The manufacturer claims that the isotopic DNA probe assay, in a population of 441 females with an overall prevalence of 20%, had a sensitivity and specificity of 87% and 96% when compared to tissue culture. They also claim that the PACE^{TM} assay, in 852 females with an average prevalence rate of 13%, had a sensitivity and specificity of 88% and 95% when compared to tissue culture. Both assays are scheduled to be released on the market in 1988. Presently there are no independent evaluations in the literature. Gen-Probe offers what appears to be the first feasible DNA probe-based assays for detecting C. trachomatis in the clinical laboratory.

Evaluation of Rapid Diagnostic Tests

As mentioned above, tissue culture isolation is the reference standard against which all nonculture tests are measured (12,125,129). To evaluate a nonculture-test, one specimen is usually collected for the nonculture-test and a second specimen for tissue culture. Results are compiled in a 2 by 2 table (125) (see table 5) and the nonculture-test's

sensitivity, specificity, predictive value (+) and predictive value (-) are calculated using tissue culture as the reference standard. Since culture is considered near 100% specific (125), any nonculture-test negative but culture positive is considered a nonculture-test false negative. These results could, however, be due to swab-to-swab variations, especially in low titer infections, or improper collection of the nonculture specimen as well as actual insensitivity of the nonculture-test (tissue culture does have the ability to amplify low titer infections). A second, perhaps more important problem in comparison studies arises when an antigen test is positive and culture is negative. Since (as discussed earlier) culture is not 100% sensitive, two possibilities exist: (1) a bogus false positive due to tissue culture failure to detect organisms detected by the nonculture-test caused by: (A) insensitivity of the tissue culture method, (B) swab-to-swab variation (especially in low titer infections), (C) poor specimen collection and/or storage of the culture specimen, (D) non-viable elementary bodies or non-infectious reticulate bodies detected by the nonculture-test, or (E) specimen toxicity to chlamydiae or tissue culture cells rendering a negative culture despite the presence of organisms; (2) a real false positive nonculture result due to: (A) technical procedure errors, or (B) crossreactivity with other microorganisms, or substances in the clinical specimens.

It is difficult, in many cases impossible, to

Table 5. Evaluation of a Non-Culture Diagnostic Test[a]

		Culture +	Culture -
New Test	+	a	b
	-	c	d

Element	Formula	Definition
Sensitivity	a/(a + c)	Ability of test to identify people who are culture-positive
Specificity	d/(d + b)	Ability of test to identify people who are culture-negative
Predictive Value (+)	a/(a + b)	Probability of being culture-positive given a positive test
Predictive Value (-)	d/(c + d)	Probability of being culture-negative given a negative test
False positives	b	Culture-negative specimens that test positive
False negatives	c	Culture-positive specimens that test negative

[a]Adapted from: Schachter, J. 1985. Immunodiagnosis of sexually transmitted disease. Yale J. Biol. Med. 58:443-452.

differentiate culture failures from real nonculture false positive results. A few investigators have performed various forms of discrepancy analysis to distinguish real false positive nonculture-tests from culture failures. Discrepancy analysis has included such procedures as testing residual culture 2-SP transport media by DFA for elementary bodies (6,51), testing residual ELISA buffer by DFA for elementary bodies (6,51,112,151), or recalling in patients, then collecting and culturing new specimens prior to treatment (71,73).

Presently the major problem in using rapid nonculture tests to diagnose C. trachomatis infections is the risk of real false positive results. This can be seen by examining the predictive value of a positive test (125,158). Schachter (125) explains that in a low prevalence population with a 3% prevalence rate of infection, a positive result using a test with 97% specificity, would only have a predictive value of 50%. The major problem with false positive findings occurs in interpreting positive results in low prevalence populations (125,129). Physicians must, therefore, realize the potential problems with screening for C. trachomatis with nonculture-tests especially in low prevalence populations. Careful laboratory quality control of nonculture chlamydial testing, is for the same reasons, absolutely necessary to control technical false positive results (144). Addiss et al. (2) recommends when a nonculture-test result is doubted (e.g. patient is likely to

have a false-positive test, or therapy could be potentially harmful) that the patient should be retested with the same nonculture-test and tissue culture.

Research

My research presented in this thesis focused on C. trachomatis detection methods. It consisted of two phases: (1) evaluation of variance in the tissue culture standard technique; and (2) comparison of detection of C. trachomatis by two new DNA probe-based assays and an established ELISA method to the tissue culture standard, carefully analyzing discrepant results.

MATERIALS AND METHOD

Culture Variation Study

Patient Populations

From 25 June 1987 to 10 September 1987, endocervical swab specimens were obtained in triplicate from 209 women (table 6). The specimens were collected from patients in two clinics at the Medical College of Virginia (MCV) in Richmond Virginia: ninety-nine women were from the MCV Hospital's adolescent clinic (AC) and 110 women were college students seen by the Virginia Commonwealth University (VCU) Health Service Clinic (UHS). All consenting patients from both clinics among whom a pelvic examination was indicated, both symptomatic patients and asymptomatic women undergoing periodic health examinations, were included in the study.

Specimen Collection, Transport, and Storage

UHS: For each patient, a physician or nurse practitioner initially cleansed the cervical os using a large rayon-tipped swab (Scoppette, Jr., Birchwood Laboratories, Prairie, Minnesota). Next, a Papanicolaou smear was collected from those patients undergoing periodic health examinations. Then, four consecutive endocervical specimens (swabs one-four) were collected using rayon-tipped

plastic-shaft swabs (Culturette, Marian Scientific, Kansas City, Missouri): swab one was not part of the study, swabs two through four were immediately placed in 2-sucrose phosphate (2-SP) transport medium (Whittaker M.A. Bioproducts, Waltersville, Maryland). The 2-SP contained 2.0 milliliter (ml) of medium per vial, antibiotics (gentamicin and nystatin), and sterile glass beads. Once collected, the 2-SP vials were refrigerated for up to nine hours and were then transported on ice to the laboratory where they were frozen at -65°C until processing. Specimens were stored between one and 14 days.

AC: The procedure was the same as in the UHS except when a Papanicolaou smear was required, it was collected after the four consecutive endocervical swabs.

Tissue Culture Processing

Specimens were processed in groups (batched) and tissue cultures were inoculated twice a week. Usually specimens were processed within three to four days, but always within two weeks of collection. An individual patient's three specimens were always cultured simultaneously. The 2-SP specimens were thawed at room temperature and one drop of an antibiotic solution (Cul-Trol™, Bartels Immunodiagnostic Supplies, Inc., Bellevue, Washington) was added to each vial. Cul-Trol™ contains streptomycin, gentamicin, and amphotericin B. Specimens were placed in styrofoam vial racks and were agitated (vortexed) for one minute (at setting four) using a multi-tube vortexer (Scientific

Manufacturing Industries (SMI), USA). Preincubated McCoy cells in 1-dram vials (Whittaker M.A. Bioproducts, Waltersville, Maryland) were placed in foam holding-racks and were pretreated with 1.0 ml of diethylaminoethyl (DEAE)-Dextran in phosphate-buffered saline (Bartels Immunodiagnostic Supplies, Inc., Bellevue, Washington) for one minute. The initial DEAE-Dextran was then decanted and a second 1.0 ml DEAE-Dextran pretreatment was performed. Using sterile 1.0 ml pipettes, the McCoy cells were next inoculated with 0.5 ml of 2-SP (after inoculation, each batch of 2-SP specimens was immediately refrozen at -65°C). Each specimen was set up in duplicate. The McCoy cells were then centrifuged between 1700 and 2200 x G at 35°C for one hour. The inoculum was decanted and 1.0 ml of filtered phosphate buffered saline (Dulbecco's Modified w/o phenol red, Ca, and Mg, Hazleton Research Products, Inc., Lenexa, Kansas), was added to each McCoy cell vial to "wash" the cells. The vials were gently swirled and decanted, followed by a second PBS wash. Next, 1.0 ml of growth media (90.0 ml Cycloheximide Overlay Medium Base supplemented with 10.0 ml Fetal Bovine Serum - Whittaker M.A. Bioproducts, Waltersville, Maryland) was added to each McCoy cell vial. The vials were incubated approximately 48 hours at 37°C. A positive control was included in each batch of cultures.

Detection of Chlamydial Inclusions

After approximately 48 hours of incubation, the growth medium was decanted from McCoy cell vials. The tissue

monolayers were immediately fixed by adding 1.0 ml of reagent-grade methanol to each vial. After approximately 10 minutes, the methanol was decanted and using forceps, one coverslip at a time was removed from the vials. When a coverslip was removed from its vial, it was allowed to air dry for 5-10 seconds. The coverslip was then dipped in fresh distilled water, blotted on absorbent paper and placed monolayer side up on a labeled microscope slide inside a moistened petri dish. Once eight coverslips were added to the petri dish, 30 ul of Microtrak™ Chlamydia trachomatis culture confirmation test kit (Syva Co., Palo Alto, California) was added to each coverslip. The petri dish was incubated for 30 minutes at 37°C. The coverslips were rinsed with distilled water, their edges blotted on a paper towel, and mounted monolayer side down in glycerol. The monolayers were examined under a fluorescent microscope (Laborlux K/D, Leitz, Germany). Culture was considered positive if one or more typical apple-green cytoplasmic inclusion(s) was/were detected. Starting approximately two weeks into the study, the number of inclusions per coverslip was counted under 10X magnification. If inclusions were numerous, the number of inclusion bodies were counted in 20 randomly chosen fields at 450X magnification.

Passage

All negative specimens were reinoculated onto a fresh McCoy cell monolayer (passed) at least once to amplify possible low titer infections. Using flamed forceps, 2-3

sterile glass beads were added to vials of duplicate cell monolayers of all negative stained specimens. Then, starting with the one minute vortexing step, the culture procedure was repeated as described in "Tissue Culture Processing" above.

Discrepancy Analysis

Increased Passage: Discordant negative specimens, from patients in whom at least one other swab was positive, were passed up to four additional times; if they remained negative, a total of five coverslips were examined.

Reculture: Those discordant specimens that stayed negative throughout the four additional passes were recultured from the original 2-SP and were again passed up to four times.

Statistical Analyses

The Chi-square test was used to test for differences between the two clinic populations.

Table 6. Populations in the culture variation study

Patient Populations	Number Of Patients	Age Mean	Age Range
University Health Service	98	23.3	18-33
Adolescent Clinic	109	16.8	11-20

DNA Probe and ELISA Versus Tissue Culture

Patient Populations

From 31 August 1987 to 22 December 1987, a total of 884 symptomatic and asymptomatic women were sampled in triplicate, 159 women from MCV's AC and 725 from VCU's UHS. All consenting women (in both clinics), for whom a pelvic exam was indicated, were eligible for the study.

Specimen Collection, Transport, and Storage

The specimen collection was the same as in the culture variation study (see "Specimen Collection, Transport, and Storage" above) except that instead of swabs 2-4 all being cultured, the following protocol was used: swab two was always used for tissue culture; swabs three and four were rotated weekly between DNA probe (Gen-Probe, Inc., San Diego, California) and Chlamydiazyme (Abbott Laboratories, N. Chicago, Illinois). Based on swab location (third or fourth position), there was no statistical difference (compared differences of binomial proportions) in ELISA or DNA probe results (sensitivities or specificities) when compared to tissue culture.

Tissue Culture: The culture specimen was collected as described in the culture variation procedure.

Chlamydiazyme: The endocervical specimen was collected according to manufacturer's instructions using their STD-EZE collection kits for women. The kit incorporates a cotton swab which, after collection, is placed into a tube with 0.1

ml of storage reagent. After collection, the specimens were immediately refrigerated, and within nine hours were transported on ice to the laboratory. Upon receipt in the laboratory, the specimens were refrigerated until processing.

DNA Probe: The endocervical specimen was collected according to manufacturer's instructions using provided dacron swabs. During the course of the study, three brands of dacron swabs were used: (1) Dacroswab^{TM} - Spectrum Laboratories, Inc., Houston, Texas; (2) Medical Packaging Corporation, Panorama City, California; and (3) Puritan swabs, Hardwood Products Co., Gaiford, Maine. The swabs were placed in 1.0 ml of transport media supplied by the manufacturer. The specimens were refrigerated and transported to the laboratory within nine hours. Once received in the laboratory, the specimens were refrigerated. Usually within 24 hours, but always within 72 hours, the specimens were individually agitated for 10 seconds, the swabs were removed, expressed, and discarded. The specimens were then frozen at -65^{o}C until processing.

Specimen Processing

Tissue Culture: Tissue cultures were performed according to the culture procedure used in the culture variation study with the following exceptions: (1) only one McCoy cell DEAE-Dextran pretreatment was performed; (2) only one PBS wash was performed; (3) the growth media used was from a different manufacturer (Chlamydia isolation medium

with cycloheximide - Bartels Immunodiagnostic Supplies, Inc., Bellevue, Washington); (4) staining of McCoy cell monolayer took place between approximately 48-72 hours; and (5) only one pass was performed on all negative primary cultures.

Chlamydiazyme Test: The test was performed according to the manufacturer's instructions. The specimens were stored at 2-8°C and were processed within five days of collection. One ml of specimen-dilution buffer was added to each transport tube containing a swab. The tubes were warmed at room temperature for 15 minutes, then agitated on a SMI multi-tube vortexer (setting four) for three cycles of 15 seconds, after which the swabs were expressed and discarded. Two hundred microliter (ul) of specimen extract and controls (positive and negative specimens supplied in the kit) were placed in the wells of plastic plates and one treated polystyrene bead (prepared by manufacturer) was added to each well. The plate was then incubated in a water bath at 37°C for one hour. Each bead was washed four times with 4-6 ml of distilled water using a hand held multi-channel washer (Pentawash). Next, 200 ul of rabbit anti-chlamydial antibody was added to each well, incubated one hour at 37°C and followed by the same washing procedure. Then 200 ul of horseradish peroxidase conjugated antibody to rabbit IgG was added to each well, incubated one hour at 37°C and washed again. Next the beads were transferred to reaction tubes (Abbott), and 0.3 ml of freshly prepared peroxidase

substrate (ortho-phenylenediamine-hydrogen peroxide) was added to each tube. The tubes were kept in the dark at room temperature for 30 minutes and the reaction was stopped by adding 1.0 ml of 1N sulfuric acid The absorbance of specimens was determined at 492 nanometers using a Quantum II spectrophotometer (Abbott, N. Chicago, Illinois). A result was considered positive if the optical density exceeded the mean of the three negative controls plus 0.1. The results of the Chlamydiazyme were recorded without knowledge of the results of the other tests.

DNA Probe Tests

PACE™ Assay System (Non-isotopic): The test was performed according to the manufacturer's instructions. The specimens were kept frozen at -65°C until processing in batches during December 1987 and January 1988. Specimens were normally run in batches of 48 which included 43 patients, three negative controls, one low positive control, and one high positive control. Once a day, prior to testing patient specimens, a DNA probe total count was performed. This included pipetting 5.0 ul of well mixed probe into 1.0 ml of elution reagent. After mixing, 300 ul was pipetted into three separate test tubes. The three tubes were tested using the total count format of a luminometer (Leader I-Berthoid™, Germany) to determine the mean relative light units (RLU's). The DNA probe's total count was required to be between 8,000,000 and 10,000,000 RLU's. The patient specimens were thawed at room temperature. Specimens were

observed for blood and graded as slightly, moderately, or grossly bloody. Using an Eppendorf repeating pipette, 100 ul of well mixed probe solution was added to five milliliter (75 x 12 mm) plastic test tubes (Starstedt, Germany). Patient specimens were individually agitated for 5-10 seconds and 100 ul was pipetted into the tubes containing the probe. The tubes were then covered with transparent tape and agitated (vortexed) on a multi-vortex mixer (VWR, Scientific, USA) for three cycles of five seconds each. To allow for DNA/rRNA hybridization, the test tube racks were placed in a 60°C water bath for one hour. Then using an Eppendorf repeating pipette, 2.0 ml of freshly prepared separation solution (containing magnetic spheres) was added to each tube. The specimens were again sealed with transparent tape and vortexed as before. The tube racks were placed in the 60°C water bath for five minutes (to allow for DNA/rRNA hybrid binding to the magnetic spheres) and were then placed on a magnetic separation base for two minutes (to allow for magnetic sphere separation). Then holding a rack onto the magnetic base, the separation solution was decanted and the tubes blotted onto absorbent paper. Next, using the Eppendorf repeating pipette, 1.0 ml of heated (60°C) wash solution was added to each tube. The specimens were sealed with plastic wrap (Reynolds), vortexed, placed on the magnetic separation base for two minutes, decanted, and blotted. A total of three wash cycles were performed. During each wash, specimen tubes

were observed for clumping of magnetic spheres. After the third wash, 300 ul of elution reagent (to elute DNA/rRNA hybrids from the magnetic spheres) was pipetted into each tube. The tubes were sealed with transparent tape, incubated for five minutes at 60°C, and then placed on the magnetic separation base for two minutes. Next, using a transfer pipette, the eluted solution was removed carefully (to avoid pipetting of magnetic spheres) and placed in newly labeled test tubes. The controls and patient specimens were then read for chemiluminescence in the Leader I. A specimen was considered positive if the average of three negative controls divided into a patient or control result gave a relative light unit ratio of 2.4 or greater. The results of the DNA probe-based assay were recorded without knowledge of the results of other tests. After performing the non-isotopic DNA probe testing, all specimens were refrozen at -65°C.

Isotopic DNA Probe Based Assay: The test was performed according to the manufacturer's instructions. Processing of the isotopic DNA probes on the same patient probe transport specimens previously used for non-isotopic probe testing occurred in February 1988. The procedure is very similar to the non-isotopic probe-based assay including the four basic steps of sample preparation, hybridization, separation and measurement. The tests differ in the following respects: (1) since the DNA probe is isotopically labeled with ^{125}I, proper laboratory safety precautions, documentation, and

disposal procedures were implemented when performing the test; (2) using a blank test tube, a daily background count of the gamma counter was performed; (3) the total count consisted of pipetting 100 ul of probe solution into a test tube and counting the tube with the processed specimens in a gamma counter; (4) only 50 ul of control and patient specimens were added to the DNA probe solution instead of 100 ul; (5) a single negative and a single moderately positive control were used instead of three negative and two positive controls; (6) the hybridization incubation step was for two hours instead of one hour; (7) the elution step was not required, therefore, after the third wash was completed, the total count tube, the two control tubes, and patient specimens were loaded in a gamma counter (1270 Rackgamma II, LKB Wallac). The specimens were each read for one minute to derive a count per minute (CPM); (8) the positive cut-off was derived by subtracting the daily background count from the total count and multiplying the difference by 0.0007. A positive specimen was one that, after having the background count subtracted, had a net sample CPM greater than or equal to the calculated cut-off.

Discrepancy Analysis

Definitions: A non-culture test that was positive with its corresponding tissue culture negative was considered an apparent false positive. A non-culture test that was negative with its corresponding tissue culture positive was considered as an apparent false negative.

Apparent False Positives: First, the stained tissue culture monolayer (which had been stored frozen at -20°C) was reviewed. If, after review, the tissue culture monolayer still appeared negative, the next step was to rerun the nonculture test(s) up to two times until two consistent results were obtained. If the nonculture test(s) remained positive, the next step was to reculture directly from the original 2-SP transport medium; only a single primary tissue culture was performed. If the culture remained negative, the remaining 2-SP was pipetted into a microcentrifuge tube (American Scientific Products) and concentrated by centrifuging 15 minutes at 12000 x G in an Eppendorf Centrifuge (model 5414). The supernatant was then removed using a sterile transfer pipette and the sediment was resuspended in 50 to 100 ul of Hank's BSS (Hazleton Research). One drop of the resuspended sediment was then placed in an etched well (in duplicate) on a fluorescent antibody slide (Clay Adams) and allowed to air dry. The dry sediment was then fixed with acetone. Next, the slides were stained with Microtrak™ Chlamydia trachomatis direct specimen test (Syva, Palo Alto, California). Using a fluorescent microscope, both wells of the slide were examined for chlamydial elementary bodies: scanning with 60X oil magnification and confirmation at 100X oil magnification. In selected instances of apparent false positive ELISA's, the residual ELISA buffer was also concentrated, and examined for chlamydial elementary bodies

using the procedure just described.

Apparent False Negatives: First the stained tissue culture monolayer (stored frozen at -20°C) was reviewed. If, after review, the tissue monolayer still appeared positive, the next step was to rerun the nonculture test(s) up to two times until two consistent results were obtained. If the nonculture test(s) remained negative, the nonculture test(s) was/were run on the patients corresponding concentrated 2-SP transport medium. If only one nonculture test was negative, all the remaining 2-SP was concentrated in a single microcentrifuge tube, but if both nonculture tests were negative, the remaining 2-SP was divided equally into two microcentrifuge tubes. The 2-SP was concentrated as described earlier. The Chlamydiazyme procedure was performed first on the culture swab removed from the 2-SP specimen. One ml of buffer was added to the swab and the method was performed as described in the Chlamydiazyme procedure (see above). If the test was negative, then the Chlamydiazyme was performed on the concentrated 2-SP sediment to which 1.0 ml of buffer was added. The non-isotopic DNA probe was performed as described above on concentrated 2-SP sediment which was resuspended in 100 ul of transport medium.

Statistical Analyses

Sensitivity, specificity, and predictive values were calculated by conventional methods (125). Chi-square analysis was used to test for differences between the two

clinic populations. McNemar's test was used to analyze for differences between tests on matched samples.

RESULTS

Culture Variation Study

Triplicate endocervical specimens from a total of 209 women, 99 from the UHS and 110 from the AC, were cultured for isolation of C. trachomatis. One set of tissue cultures from each clinic, was considered toxic (non-interpretable due to contamination) in both the primary and pass cultures so these patients were excluded from the study. This left a total of 207 patients examined (see table 6).

A patient was considered positive for C. trachomatis infection if one or more of three cultures demonstrated characteristic cytoplasmic inclusions. The combined prevalence from both clinics was 15.9% (table 7). The prevalence was 8.1% in the UHS and 22.9% in the AC, showing a statistical significant difference (p=.004) between the prevalence rates in the two clinics.

Table 8 summarizes the primary culture results (includes only the initial culture and one pass) categorized by sequential swab combinations. Of the 207 women examined, 174 (84.1%) were negative in all three swabs, and 33 (15.9%) women were positive on at least one swab with 24 positive in all three swabs and nine positive with various patterns of discrepant positive and negative results.

The chlamydial isolation patterns of the nine positive patients with inconsistent tissue culture results are presented in table 9. The discrepancies were analyzed in a two step process. First, negative specimens were passed in culture up to four times. Most of these results, therefore, include three extra culture passes (normally only one pass is performed) of the negative discrepant specimens. The exceptions are: case 1 where specimen four (the third swab tested) did not have a first pass coverslip to stain and case 4 where specimen four was toxic beginning with the primary culture. Multiple passes yielded only one additional positive culture; in case 6, specimen 3 became positive at the second pass (the third coverslip examined).

The second step of discrepancy analysis was to reculture those discrepant negative specimens that remained negative throughout the original cultures' four passes. Table 10 summarizes the results of the nine discrepant patients after reculture. Reculture was positive (all in the initial culture, none in additional passes) in four of the 12 negative discrepant specimens (the fourth specimen in cases 1, 4, 6, and 7), resolving 3/9 culture (swab-to-swab) discrepancies (cases 1, 4, and 6). This left 3/25 (.12) culture (swab-to-swab) discrepancies in the high prevalence AC clinic and 3/8 (.38) culture (swab-to-swab) discrepancies in the lower prevalence UHS. The difference between the discrepancy rates of the two clinics was not statistically significant ($p>.05$) although this may be due to the low

number of patients in the study. Also recultured were seven low inclusion positive specimens. Three remained positive and four converted to negative. This is not surprising because these were low titer positive specimens that had an extra freeze-thaw cycle during which the chlamydiae may have lost viability (155).

As discussed earlier (in "Material and Methods") section, starting approximately one month into the study, the number of inclusion bodies per coverslip were counted. Results of inclusion body counts include only those cases where the counts were performed on the initial cultures of all three specimens and do not include counts from passes or recultures. Table 11 provides the inclusion count results of the six positive cases with unresolved inconsistent cultures. All six cases had low titer infections ranging from one to 24 inclusions per positive coverslip with a mean inclusion count of 6.6. Table 12 presents data that indicates the second swab (first cultured) had higher inclusion counts per coverslip than the third or fourth swabs (second and third cultured) in the majority of cases examined, but this difference, too, was not significant.

The detection rate of total positive patients isolated by each individual swab is shown in table 13. The second swab submitted (the first of three swabs examined) showed the best recovery, 31/33 (.94) positive patients, when compared to 29/33 (.88) for the third swab, and 27/33 (.82) for the fourth swab. If recultures were included, both the

second and fourth swabs detected 31/33 (.94) of the C. trachomatis infections isolated. The third swab, however, did not improve with reculture detecting 29/33 (.88). A significant cumulative effect was not seen by culturing three swabs instead of one. Adding two more swabs detected only 2/33 (.06) more positive patients.

Table 7. Prevalence of *Chlamydia trachomatis* in the culture variation study.

Patient Populations	Positive	Negative	Total
University Health Service[a]	8	90	98
Adolescent Clinic[b]	25	84	109
Totals	33	174	207

[a]Prevalence rate 8/98 (.08)

[b]Prevalence rate 25/109 (.23)

Difference between rates statistically significant p=.004

Table 8. Primary culture results categorized by sequential swab combinations

Combinations			Primary Culture
Swab 2	Swab 3	Swab 4	Results
-	-	-	174
+	+	+	24
+	-	-	2
+	+	-	4
+	-	+	1
-	+	+	1
-	+	-	0
-	-	+	1

Primary cultures all three swabs positive 24/33 (.73)

Primary cultures whose sequential swabs inconsistent 9/33 (.27)

Table 9. Chlamydial isolation from nine patients whose sequential samples produced inconsistent results

Case #	Clinic	Swab 2	Swab 3	Swab 4
1	AC	+	+	-[a]
2	AC	-	-	+
3	AC	+	-	-
4	AC	+	+	T
5	AC	+	-	+
6	UHS	+	+[b]	-
7	UHS	+	-	-
8	UHS	+	+	-
9	UHS	-	+	+

[a]No coverslip in pass vial

[b]Became positive at second pass (3rd coverslip)

T = culture uninterpretable due to destruction of McCoy cell monolayer

Table 10. Results after reculture of the nine patients whose primary cultures produced inconsistent results

Case #	Clinic	Swab 2	Swab 3	Swab 4	Resolved
1	AC	NR	NR	+[a]	Yes
2	AC	-	-	-[b]	No
3	AC	-[b]	-	-	No
4	AC	NR	NR	+[a]	Yes
5	AC	+	-	+	No
6	UHS	NR	NR	+[a]	Yes
7	UHS	-[b]	-	+[a]	No
8	UHS	NR	+	-	No
9	UHS	-	-	NR	No

[a]Primary culture negative turned positive

[b]Primary culture positive turned negative

NR = Not Recultured

3/25 (.12) discrepancies in AC

3/8 (.38) discrepancies in UHS

Difference between discrepancy rates is not significant ($p>.05$)

TABLE 11. Original inclusion body counts per coverslip of unresolved inconsistent results

Case #	Clinic	Swab 2	Swab 3	Swab 4
2	AC	-	-	+ (2)
3	AC	+ (2)	-	-
5	AC	+ (1)	-	+ (1)
7	UHS	+ (15)	-	-
8	UHS	+ (24)	+ (1)	-
9	UHS	-	+ (2)	+ (11)

( ) Number of inclusions

Mean inclusion count per positive coverslip = 6.6

Table 12. Comparison of primary culture inclusion body counts per coverslip for all swabs on 17 patients

Swab Comparison		Inclusion Counts		
A	B	A>B	A<B	A=B
Swab 2	Swab 3	10/17	5/17	2/17
Swab 2	Swab 4	10/17	6/17	1/17
Swab 3	Swab 4	7/17	5/17	5/17

Table 13. Specific "swab" sensitivity

Adolescent clinic positive patients

Swab	Primary	Repeat
2	24/25 (.96)	24/25 (.96)
3	22/25 (.88)	22/25 (.88)
4	22/25 (.88)	24/25 (.96)

University health service positive patients

Swab	Primary	Repeat
2	7/8 (.88)	7/8 (.88)
3	7/8 (.88)	7/8 (.88)
4	5/8 (.63)	7/8 (.88)

Total positive patients

Swab	Primary	Repeat
2	31/33 (.94)	31/33 (.94)
3	29/33 (.88)	29/33 (.88)
4	27*/33 (.82)	31/33 (.94)

*One culture did not have a coverslip to pass and a second culture was uninterpretable

DNA Probes and ELISA Versus Tissue Culture

Comparison of NIP and ELISA Results

Triplicate endocervical specimens from a total of 884 women, 159 from AC and 725 from UHS, were examined for C. trachomatis. The second swab collected was always used for tissue culture; the third and fourth swabs were rotated weekly between ELISA and the DNA probe (see "Materials and Methods"). The DNA probe specimens were tested by both a non-isotopic and an isotopic probe (NIP and IP). Fourteen of 884 (1.6%) patient results, 2/159 (1.3%) from AC and 12/725 (1.7%) from UHS, were excluded from the study for the following reasons: (1) eight for improper collection or storage of one of the three test specimens, (2) five because the DNA probe specimen transport tube contained a double volume of transport medium, and (3) one because the culture was uninterpretable in both the initial and pass culture. This left a total of 870 patients tested by all four methods.

Ninety-five patients had Chlamydia isolated by tissue culture for a prevalence of 95/870 (10.9%). Table 14 shows the prevalence rates of C. trachomatis in both patient populations. The prevalence rate was 26/157 (16.6%) in the AC and 69/713 (9.7%) in the UHS. As in the culture variation study, there is a statistical difference (p=.015) between the prevalence rates of the two clinics.

The three nonculture methods' performance compared to the tissue culture reference standard is summarized by

showing each test's sensitivity, specificity, predictive value of a positive, and predictive value of a negative in the AC (table 15), in the UHS (table 16), and in both clinics combined (table 17). The overall sensitivities of ELISA (90.5%), NIP (89.5%), and IP (86.3%) were similar with the later tested IP being slightly lower. All three nonculture tests had slightly, but not significantly lower sensitivities in the AC than in the UHS. There are, however, larger differences in the overall specificities of the three tests with the NIP having a statistically lower specificity of 93.2% than ELISA 98.2% ($p>.05$) and IP 97.8% ($p>.05$). Both DNA probes had slightly lower specificities in the AC than in the UHS where ELISA remained stable in both groups.

Analysis of apparent nonculture false negative samples

ELISA only false negatives: Table 18 summarizes the nonculture false negative results for ELISA and NIP (IP discrepancy analysis will be presented separately below). Four patients were culture positive, NIP positive, and ELISA false negative. When the four ELISA specimens were repeated, one specimen result converted to positive (attributed to inter-run variability). Of the three patients whose ELISA specimens remained negative, all showed a positive result when the ELISA method was performed on the corresponding concentrated residual 2-SP culture transport specimens (indicating probable swab-to-swab variation).

NIP only false negatives: In five cases, culture and

ELISA were positive and the NIP samples were negative. The five NIP samples were repeated and all remained negative. Next, the NIP method was performed on four of the five corresponding 2-SP specimens (one missing) with three giving positive results. Thus, these three cases are also examples of probable swab-to-swab variation.

ELISA and NIP false negatives: There were also five culture positive patients that were negative for both the ELISA and NIP tests and all five when retested were negative for both tests. ELISA and NIP methods were then performed on separate equal portions of corresponding 2-SP specimens. Both ELISA and NIP showed two positive results (on the same two specimens), once again demonstrating probable swab-to-swab variation.

Thus, five of the nine ELISA and five of the 10 NIP false negative tests, tested positive when the procedures were performed on the same patients' corresponding 2-SP specimens. This indicates that the culture 2-SP medium probably contained more chlamydial elementary bodies than the ELISA's and/or NIP's transport medium. Consequently about half of the nonculture false negative results are due to swab-to-swab variation in collection. This correlates with the culture variation study in that swab 2 (first swab cultured) detected the most positive patients and also had the highest number of inclusions per coverslip in the majority of cases. Table 19 presents evidence that the chlamydial infections were low titer among 13 of the 15

patient cases (except cases 1 and 11) demonstrating various patterns of nonculture test(s) false negative results. Nine of the tissue culture positive specimens were associated with low initial inclusion counts, ranging from 2 to 15 inclusions per coverslip; four other cases were not positive until the tissue culture specimens were passed. Swab-to-swab variation thus correlates with low titer infections.

Analysis of apparent nonculture false positive samples

The major difference found in using nonculture methods instead of tissue culture is an increase in false positive results. These are a combination of actual false positive test results and culture failures. Table 20 summarizes the nonculture false positive results of ELISA and NIP (IP discrepancy analysis will be presented separately below).

ELISA only false positives: Ten patients were culture negative, NIP negative, and ELISA false positive. Nine of the 10 false positive ELISA specimens (one ELISA specimen and its corresponding 2-SP specimen were unavailable for retesting) were repeated with six converting to negative (attributable to inter-run variability). Of the three patients in which the ELISA specimens remained positive, one was reculture-positive from its corresponding 2-SP specimen, indicating that the ELISA was definitely associated with a true chlamydial infection (culture failure) and chlamydial elementary bodies were seen in another patient's corresponding 2-SP specimen (probable culture failure). This only leaves two ELISA-only false positive results which

are inconclusive as to whether they are true Chlamydia-infections or actual false positive tests.

NIP only false positives: There were 49 culture and ELISA negative, NIP positive patients. After repeating, 22 NIP positive results converted to negative (attributable to inter-run variability). Of the 27 remaining NIP-only positive patients, 23 had corresponding 2-SP specimens available for reculture and 24 for DFA testing. All 23 were reculture negative, but one of the 24 was DFA positive (probe transport medium was also grossly bloody). Twenty-one of the 26 remaining NIP-only positive patients had probe specimens that correlated with either gross blood in their specimens or clumping of magnetic spheres throughout all three wash steps of the NIP procedure.

Twelve of the 14 NIP specimens that were graded as grossly bloody with corresponding negative cultures were false positives. The other two grossly bloody specimens that were NIP negative had RLU ratios of 2.3 and 2.1, just below the manufacturer's 2.4 cut-off. Those NIP specimens graded as slightly bloody or moderately bloody were not, however, associated with a significant number of consistently false positive results. Of 81 culture negative patients with slightly bloody NIP specimens, only one NIP specimen was repeatedly positive, but it also was associated with clumping of magnetic spheres (see below); of 13 culture negative patients with moderately bloody NIP specimens, only one was repeatedly positive. Moderately bloody NIP

specimens did, however, have higher negative RLU ratios than negative NIP specimens without blood and slight or moderate blood was present in five of the 22 NIP false positive specimens that converted to negative on repeat. Therefore, grossly bloody specimens caused false positive NIP results and blood in varying amounts may have been responsible for some of the inter-run variation seen.

During testing of some NIP specimens, the magnetic spheres clumped together during the separation and wash steps. All nine culture negative patients in which clumping of magnetic spheres was observed throughout all three wash steps of the NIP procedure, demonstrated false positive results. In the six NIP specimens in which clumping was observed only during the first wash, one was false positive, but upon repeat was negative and of the six NIP samples which clumped throughout two washes, only one was positive, but it was also grossly bloody.

Gen Probe, Inc. claims to have made changes in their separation and wash reagents and to the luminometers signal measurement time, to prevent non-specific chemiluminescence due to the presence of blood and to minimize the clumping of magnetic spheres. The clumping is thought to interfere with the washing of unhybridized DNA probe out of the reaction tube and thus leads to false positive results. Following testing at Gen Probe, the grossly bloody specimens and those specimens which clumped through the three washes were returned to us. These specimens were retested using Gen

Probe's updated NIP kit and the results are as follows: (1) of the 13 bloody specimens (12 grossly and one moderately) associated with NIP false positive results, only one retested positive, with a low 2.5 ratio; (2) none of the nine specimens in which clumping of magnetic spheres (throughout all three wash steps of the NIP procedure) had been associated with false positive results, retested positive; only one specimen showed clumping and it was just in the first wash step.

ELISA and NIP false positives: There were also four culture negative patients that were positive by both ELISA and NIP. All four ELISA and NIP tests remained positive when repeated. Reculture of the four patients' corresponding 2-SP specimens were all negative, but DFA analysis of three of these four 2-SP samples demonstrated chlamydial elementary bodies (probable culture failures). One of the 2-SP specimens did not have enough media left to concentrate, so this patient's residual ELISA buffer was concentrated and it was DFA positive (probable culture failure). Thus, in all cases where both nonculture tests were repeatedly positive, the culture standard was found to be defective.

Culture "sensitivity": If one considers a positive reculture of chlamydial transport media, and the detection of chlamydial elementary bodies in the 2-SP media and/or ELISA specimen buffer as culture failures, then primary tissue culture detected 95 of 102 (only 93.1%) of the

detectable chlamydial infections. Thus, tissue culture sensitivity is only slightly higher and insignificantly than the sensitivities of ELISA (90.2%) and NIP (88.2%) as recalculated after discrepancy analysis (see table 21).

Comparison of IP and NIP results

Table 22 summarizes the various patterns comparing the IP and NIP results (both were performed on the same probe transport samples) to tissue culture. The sensitivities of the two procedures were similar with NIP being slightly more sensitive 85/95 (89.5%) than IP 82/95 (86.3%) (see table 17). The major difference between the two tests is their specificities (IP 97.8% and NIP 93.2%) with the IP agreeing with 758 and the NIP agreeing with 722 of the 775 culture negative patients. The lower specificity of the NIP procedure can be attributed to three factors mentioned above: (1) increased inter-run variability, (2) false positive reactions due to grossly bloody specimens, and (3) a greater number of false positive results due to clumping of magnetic spheres (the IP test kit already included some of the manufacturer's changes made to correct for clumping).

Analysis of apparent false negative results

The five NIP-only false negative patients were all also negative by IP (see table 18.). This is also true of the five NIP and ELISA false negative patients. Three specimens that were NIP positive, initially tested IP negative, however, two of these retested positive (attributable to

inter-run variability). So, the sensitivities of the methods remain nearly equal after resolution of discrepant negative specimens.

Analysis of apparent false positive results

There were a total of 17 IP false positive specimens (see table 20.). Nine were IP-only with eight converting to negative on repeat (due to inter-run variation); one was an NIP negative but ELISA and DFA positive (most likely a true chlamydial infection). Four of the 17 IP false positive specimens were co-positive with NIP. Two of these positive results were from the nine samples that showed clumping of magnetic spheres throughout the three wash steps. In the IP procedure, seven of the samples did not clump magnetic spheres in the wash steps, but in two samples clumping was still observed in washes 1 and 2 and both were positive. The other two co-positive IP and NIP samples had no visible reason to be false positive and were repeat-culture and DFA negative. None of the 22 NIP specimens that converted to negative were positive by IP. All grossly bloody specimens that were NIP false positive were negative by IP, as would be expected, since the blood does not interfere with the detection of the isotopic label. Lastly, the four culture negative, ELISA and NIP positive patients were also IP positive, providing further evidence that these patients most likely had chlamydial infections.

Table 14. Prevalence of Chlamydia trachomatis based on tissue culture in two clinical populations

Clinic	Asymptomatic[a] +/Total	%	Symptomatic[a] +/Total	%	Total[b] +/Total	%
University Health	22/256	8.6	47/457	10.3	69/713	9.7
Adolescent	5/45	11.1	21/112	18.8	26/157	16.6
Total	27/301	9.0	68/569	12.0	95/870	10.9

[a]P = NS For differences between asymptomatic and symptomatic patients within clinics

[b]P = .015 For difference between clinic total prevalence rates

Table 15. Nonculture and culture results in the adolescent clinic

		Culture +	Culture -
ELISA	+	22	2
	-	4	129

Sensitivity = 84.6%
Specificity = 98.5%
Predictive Value (+) = 91.7%
Predictive Value (-) = 97.0%

		Culture +	Culture -
NI Probe	+	21	10
	-	5	121

Sensitivity = 80.8%
Specificity = 92.4%
Predictive Value (+) = 67.7%
Predictive Value (-) = 96.0%

		Culture +	Culture -
I Probe	+	21	7
	-	5	124

Sensitivity = 80.8%
Specificity = 94.7%
Predictive Value (+) = 75.0%
Predictive Value (-) = 96.1%

Table 16. Nonculture and culture results in the university health service

		Culture +	Culture -
ELISA	+	64	12
	-	5	632

Sensitivity = 92.8%
Specificity = 98.1%
Predictive Value (+) = 84.2%
Predictive Value (-) = 99.2%

		Culture +	Culture -
NI Probe	+	64	43
	-	5	601

Sensitivity =92.8%
Specificity = 93.3%
Predictive Value (+) = 59.8%
Predictive Value (-) = 99.2%

		Culture +	Culture -
I Probe	+	61	10
	-	8	634

Sensitivity =88.4%
Specificity = 98.4%
Predictive Value (+) = 85.9%
Predictive Value (-) = 98.8%

Table 17. Total nonculture and culture results in both clinics combined

		Culture +	Culture -	
ELISA	+	86	14	Sensitivity = 90.5% Specificity = 98.2% Predictive Value (+) = 86.0% Predictive Value (-) = 98.8%
	-	9	761	

		Culture +	Culture -	
NI Probe	+	85	53	Sensitivity =89.5% Specificity = 93.2% Predictive Value (+) = 61.6% Predictive Value (-) = 98.6%
	-	10	722	

		Culture +	Culture -	
I Probe	+	82	17	Sensitivity = 86.3% Specificity = 97.8% Predictive Value (+) = 82.8% Predictive Value (-) = 98.3%
	-	13	758	

Table 18. Resolution of apparent nonculture false negative samples

Culture Positive Patterns			Number of Patients with Pattern	Rerun ELISA[a]	Rerun NIP[a]	Rerun IP[a]	ELISA on 2-SP[a]	NIP on 2-SP[a]
ELISA	NIP	IP						
+	+	+	80	ND	ND	ND	ND	ND
+	+	-	1	ND	ND	1/1	ND	ND
-	+	+	2	1/2	ND	ND	1/1	ND
-	+	-	2	0/2	ND	1/2[b]	2/2	ND
+	-	-	5	ND	0/5	0/5[b]	ND	3/4[c]
-	-	-	5	0/4[c]	0/5	0/5[b]	2/5	2/5

[a]Denominator = the number of samples tested

[a]Numerator = the number of positive samples tested

[b]IP was not tested on residual 2-SP medium

[c]One sample not available for testing

ND = not done

Table 19. Inclusion counts per coverslip and discrepancy analysis of nonculture test(s) false negative patient specimens

Case #	Culture[a]	ELISA	NIP	IP	Comments
1	[b]TN/NP	-[c]	+	+	ELISA retested (+)
2	4/NP	-	+	+	ELISA (+) on 2-SP
3	10/NP	-	+	-[c]	ELISA (+) on 2-SP
4	-/+	-	+	-[d]	ELISA (+) on 2-SP
5	2/NP	+	-	-	NIP (-) on 2-SP
6	2/NP	+	-	-	2-SP missing
7	10/NP	+	-	-	NIP (+) on 2-SP
8	2/NP	+	-	-	NIP (+) on 2-SP
9	15/NP	+	-	-	NIP (+) on 2-SP
10	2/NP	+	+	-[c]	IP retested (+)
11	-/+	-	-	-	NIP and ELISA (+) 2-SP
12	87/NP	-	-	-	NIP and ELISA (+) 2-SP
13	4/NP	-	-	-	NIP and ELISA (-) 2-SP
14	-/+	-	-	-	NIP and ELISA (-) 2-SP
15	-/+	-	-	-	NIP and ELISA (-) 2-SP

[a]The numerator indicates the number of inclusion bodies on the initial culture coverslip unless the culture was negative. The denominator indicates either that a culture was not passed (NP) or that the passed culture was positive (+).

[b]TN = To numerous to count

[c](-) retested positive

[d]Gross blood may have aided the positive NIP test and explain why the IP test is negative.

Table 20. Resolution of apparent nonculture false positive samples

Culture (-) Patterns			Number of Patients with Pattern	Rerun ELISA[a]	Rerun NIP[a]	Rerun IP[a]	Reculture of 2-SP[b]	DFA of 2-SP and/or ELISA Buffer[b]
ELISA	NIP	IP						
-	-	-	704	ND	ND	ND	ND	ND
+	-	-	9	6/8[c]	ND	ND	1/2	0/1
+	-	+	1	1/1	0/1	1/1	0/1	1/1
-	+	-	45	ND	22/45[d]	ND	0/20[e]	1/21
-	-	+	8	ND	0/0	8/8	ND	ND
-	+	+	4	ND	0/4	0/4	0/3[f]	0/3
+	+	+	4	0/4	0/4	0/4	0/4	4/4

[a]Numerator = the number of false positive samples that converted to negative upon repeat; denominator = the number of samples tested.
[b]Numerator = the number of positive samples; denominator = the number of samples.
[c]One of the ELISA-only patient's 2-SP and ELISA samples were unavailable for retesting.
[d]Two of the 22 NIP negative repeats had only one repeated test instead of two, due to sample depletion.
[e]Two 2-SP samples were unavailable for reculture and DFA analysis and one had only enough medium for DFA analysis.
[f]One 2-SP sample was unavailable for reculture and DFA analysis.

Table 21. Comparison of performance characteristics using two different definitions for infection

Definition of Positive	Test Procedure	Sens (%)	Spec (%)	Predictive Value (+) (%)	Predictive Value (-) (%)
Cell culture positive	Culture	95/95 (100)	775/775 (100)	95/95 (100)	775/775 (100)
	ELISA	86/95 (90.5)	761/775 (98.2)	86/100 (86.0)	761/770 (98.8)
	NIP	85/95 (89.5)	772/775 (93.2)	85/138 (61.6)	722/732 (98.6)
	IP	82/95 (86.3)	758/775 (97.8)	82/99 (82.8)	758/771 (98.3)
Cell culture positive, or elementary bodies in culture 2-SP media or in ELISA buffer	Culture	95/102 (93.1)	768/768 (100)	95/95 (100)	768/775 (99.1)
	ELISA	92/102 (90.2)	760/768 (99.0)	92/100 (92.0)	760/770 (98.7)
	NIP	90/102 (88.2)	720/768 (93.8)	90/138 (65.2)	720/732 (98.4)
	IP	87/102 (85.3)	756/768 (98.4)	87/99 (87.9)	756/771 (98.1)

Table 22. Comparison of NIP and IP to tissue culture

Pattern of Results			Clinic		
Culture	NIP	IP	AC	UHS	Total
+	+	+	21	61	82
+	-	+	0	0	0
+	-	-	5	5	10
+	+	-	0	3[a]	3
-	+	+	2	6	8
-	-	+	5[b]	4[b]	9
-	+	-	8	37	45
-	-	-	116	597	713

[a]See table 18 for discrepancy analysis of the three IP only false negatives

[b]Eight of the nine IP only false positives converted to negative upon repeat. The IP one repeat positive was also ELISA positive and DFA positive on 2-SP.

DISCUSSION

Culture Variation Study

Tissue culture, the reference method for the detection of C. trachomatis infections (125,129), has two important attributes: (1) its ability to allow for multiplication of single elementary bodies into detectable intracytoplasmic inclusions and (2) its near 100% specificity rate. Tissue culture is, however, considerably less than 100% sensitive and culture negative patients may still harbor the microorganism (12,125,129). When triplicate specimens from each patient were collected and cultured simultaneously, the results in this study show swab-to-swab variation (unresolvable discrepant negative culture specimens) in six of 33 (18.2%) infected patients. Sensitivity differences seen in evaluations comparing tissue culture and nonculture methods that are less than this level of variation may not be due to differences in the methods as detectors of Chlamydia; in a majority of cases they probably just reflect swab-to-swab variation inherent in specimen collection. The swab-to-swab variation was greater in the lower prevalence UHS (3/8) than in the higher prevalence AC (3/25) patient population. A possible explanation for this trend is that,

since the lower prevalence UHS population contains a greater number of women with low titer infections, swab-to-swab variation is more apt to occur in low titer infections. This conjecture is supported by the observation that all six positive patients with unresolvable discrepant results had low chlamydial inclusion counts with a mean of 6.6 inclusions per positive coverslip. The difference in the discrepancy rates between the two clinics was not, however, statistically significant; this failure to reach significance may be due to the low number of Chlamydia positive patients in each patient group.

Other investigators have assessed the sensitivity of the tissue culture technique by culturing multiple specimens from the same site at the same time (22,24,94,136). Two of these studies (24,136) reported a sequential effect (later swabs showing greater sensitivity than earlier swabs). A significant cumulative effect (showing that the processing of multiple swabs increases isolation rate) was also reported in these and one other study (22,24,136). None of these studies used what is currently considered the optimal culture method: inoculation of cycloheximide-treated McCoy cells in vials and detection of inclusions by fluorescent monoclonal antibodies (12,125,133). If the sensitivity of the culture method used to evaluate multiple specimens is insensitive due to deficiencies in collection, storage, or processing, one would predict a greater number of discrepant results (94). Some of the factors which should be

considered when reviewing the above studies include: (1) whether the cervical os was cleansed prior to culturing (24); (2) whether cotton swabs with wooden shafts (22,24) or calcium alginate swabs (136) were used for culture [both types of swabs have been shown to be toxic to chlamydiae (78,85)]; (3) whether the first swab-specimen collected was used for additional tests besides chlamydial culture [i.e. for Neisseria gonorrheae (GC) culture and/or gram stain (24,136)], a practice that may decrease the chlamydial isolation rate (136); (4) whether irradiated cells (24) were used instead of cycloheximide-treated cells [cycloheximide-treated McCoy cells have been shown to support a greater number of inclusions than irradiated cells (25,69,115)]; (5) whether microtiter plates (24) were used rather than shell vials (as discussed below, microtiter plate cultures may be less sensitive); (6) whether a detection method other than fluorescent monoclonal antibodies (FA) (22,24,94,136) was employed for staining cell monolayers [FA stain is considered the most sensitive detection method available (125,145,147)]; and (7) whether or not a pass of negative cultures is performed (only reference 136 specified using a culture pass, but only if the initial culture was toxic); Dunlop et al. (22) did not even provide an account of the culture procedure used in their study.

The results from this study failed to show either a sequential effect or a major cumulative effect. The second swab (the first of three swabs examined) showed the best

recovery 31/33 (93.9%). The second swab also had higher inclusion counts than the third or fourth swab in 10/17 (59%) cases. There was only a minor cumulative effect; adding two more swabs detected only 2/33 (6.1%) more Chlamydia infected patients than the first swab examined detected alone. These results concur with those of Munday et al. (94). They reported that two additional cervical swabs increased the isolation rate by only 2%, and they did not see a sequential effect (the chance of the first swab or third swab having the most inclusions was the same). In a similar study of a nonculture detection technique, Hernandez et al. (46) performed an ELISA (Chlamydiazyme, Abbott) on multiple cervical swabs in two patient populations. They also reported no substantial sequential or cumulative effects.

These results disagree with those presented by Embil et al. (24). These authors cultured five consecutive cervical swabs and reported that the fourth and fifth swabs isolated 80-82% of the Chlamydia infected patients compared to 67-69% from the first and second swabs. They also found that two swabs increased the number of detectable cases by as much as 44.7%. However, as noted above, the culture method used was insensitive due to: not cleaning the cervical os, using cotton swabs with wooden shafts, use of the first swab for GC culture and gram stain as well as Chlamydia culture, McCoy cell irradiation, use of microtiter plates, and failure to use FA inclusion detection. The variation

between specimens that Embil et al. observed may, therefore, be misleading. Dunlop et al. (22) also observed a cumulative effect in performing triple cultures of various female sites. From the cervical site, they reported that the first swab yielded 59% of the Chlamydia infections detected, the second swab an additional 14%, and the third swab an additional 8%. However, their results cannot be analyzed because the sensitivity of their culture method is unknown as they did not describe their culture technique. They did use cotton swabs with wooden shafts that they reported as being nontoxic to chlamydia. This assertion has been contradicted by other workers (78,85). Singal et al. (136) reported cumulative and sequential effects when culturing two consecutive swabs from male urethral specimens. The first specimen, however, was used for both a GC culture and an occasional gram stain, as well as for chlamydial culture. This, as noted by the authors themselves, may have decreased the sensitivity of the first swab for isolating C. trachomatis.

Jones et al. (57) reported that increasing the number of culture passages to four (five coverslips examined) resulted in a 35% increase in chlamydial isolation after the final two passes. In the study presented here, not a single initial negative specimen became positive after a single pass. Also, multiple passes of discrepant negative specimens of known infected patients detected only one more positive swab (on a second pass), while direct reculturing

of 2-SP transport media detected four more positive swabs resolving 3/9 (33%) swab-to-swab discrepancies. It appears Jones and co-workers' culture method may lack sensitivity as suggested in a recent publication by Schachter and Martin (133). They report that multiple blind passages are not necessary to maximize the chlamydial isolation rate in a vial system, but may be necessary in the less sensitive microtiter plate system (Jones et al. used 96 well microtiter plates). Instead of multiple passes, a more efficient method to increase sensitivity of chlamydial isolation in female patients may be to culture a pooled endocervical and urethral specimen (57,80).

In summary: (1) even when using an optimal culture method, swab-to-swab variation occurs in detection of C. trachomatis and appears to be associated with low titer infections; (2) a sequential trend was not seen with the first of three swabs cultured showing the best detection of chlamydial infections; (3) adding two more swabs detected only 6.1% more chlamydial infections; and (4) a single pass of negative patient specimens and multiple passes of discrepant negative specimens of infected patients were not effective in increasing isolation of C. trachomatis.

Based on this study's results to be cost effective and sensitive, it is recommended clinical laboratories that perform tissue culture sample a single swab (the second endocervical swab collected), inoculate McCoy cells in shell vials, and use FA for detection of inclusions. If a well

trained medical technologist thoroughly reviews the entire coverslip for inclusion bodies, a pass would not be required (in populations with lower prevalence rates a pass may be more effective). Deleting routine passage will allow for increased screening of patients at risk for chlamydial infections, with no increase in cost, and will detect more infected patients.

DNA Probes and ELISA Versus Tissue Culture

For the detection of C. trachomatis infections, tissue culture is the reference standard against which all nonculture tests are measured (12,125,129). A sensitive and specific culture method is necessary to properly evaluate nonculture methods. As Schachter (129) has pointed out, an insensitive culture method may cause an insensitive nonculture method to look sensitive, however, it could also make a sensitive nonculture method to appear nonspecific. The tissue culture method used in this study is considered the most sensitive method currently available (12,125,133).

This study evaluated the comparative usefulness of culture, ELISA, and two soon to be released DNA probes (NIP and IP) for the detection of chlamydial infection in 2,610 samples from 870 patients in two populations. The results of this study showed that there was no significant difference in true positive rates among ELISA (90.5%), NIP (89.5%), and IP (86.3%) when compared to tissue culture. This is reflected by the overall similarity of each method's predictive value of a negative with ELISA 98.8%, NIP 98.6%, and IP 98.3%. The ELISA's sensitivity of 90.5% compares favorably to the median of 85% (range 60-96%) reported in women of intermediate-prevalence populations as compiled from 12 studies by Stamm (141). Presently there are no independent published studies of the DNA probes with which to compare sensitivities.

Although the sensitivities of the three tests were

similar, there was a significant difference between the overall specificity of NIP (93.2%) and the specificities of both ELISA (98.2%) and IP (97.8%). This is reflected in each method's predictive value of positive (PV(+)). An NIP positive had only a 61.5% chance of being a true positive, while both ELISA and IP had much higher chances of 86.0% and 82.8% respectively. The ELISA specificity of 98.2% compares favorably with the median 97% (range 93-98%) of the reports found in women in intermediate-prevalence populations as compiled from 12 studies by Stamm (141). As discussed in the results section, the major reasons for NIP having a lower specificity and PV (+) than seen in ELISA and IP can be attributed to three factors: (1) greater inter-run variability, (2) false positive reactions due to grossly bloody specimens, and (3) false positive reactions caused by clumping of magnetic spheres.

Provided that both the culture and nonculture procedures are performed correctly, a false negative nonculture test result could occur from either swab-to-swab variation or from insensitivity of a nonculture test in comparison to tissue culture. In 13/15 (86.7%) cases, culture positive patients with one or more discrepant nonculture test result(s) were associated with what appear to be low titer chlamydial infections. This correlates with the results seen in the culture variation study described above in which the six patients that showed culture variation also had low titer chlamydial infections. The

results of these two studies strengthens the argument that swab-to-swab variation is more apt to occur in collection of specimens from patients with low titer infections.

Further evidence that shows swab-to-swab variation was responsible for at least half of the nonculture tests' false negative results was that 5/9 ELISA and 5/10 NIP false negative patients yielded positive results when the nonculture procedure(s) was/were assayed directly on the residual 2-SP transport medium of the culture-positive swabs. This observation suggests that a greater number of chlamydial organisms were in the culture positive 2-SP medium than in the false negative nonculture tests' transport media. Referring again to the culture variation study, one notes that the second swab (first swab examined) had the highest chlamydial isolation rate and, in the majority of cases examined, the greatest number of chlamydial inclusion bodies per coverslip. In the study comparing nonculture methods, the first swab sampled was always used for culture. Subsequent positive nonculture tests on the transport medium of the first swab sampled, in cases where the nonculture tests had been negative on samples from later swabs, are also evidence that the earliest swab may be most sensitive.

The other cause of false negative nonculture results is decreased sensitivity of nonculture tests compared to tissue culture. This is probably the explanation for many of the false negative nonculture results in which the nonculture

technique was also negative on the corresponding residual 2-SP transport specimens. As stated above, tissue culture does have the ability to allow for multiplication of single elementary bodies into detectable intra-cytoplasmic inclusions. Taylor-Robinson et al. (151) reported, in comparing titration of four laboratory strains of C. trachomatis, Chlamydiazyme was at least 100-fold less able to detect Chlamydia than tissue culture. Similar titration studies are not published for either DNA probe method.

Thus, false negative nonculture tests are probably caused by a combination of: (1) swab-to-swab variation associated with low titer infections; (2) relative insensitivity of the nonculture test in low titer specimens compared to tissue culture; and (3) inter-run variability of the nonculture method (one ELISA false negative specimen repeated positive). However, in the populations evaluated, there was no difference between the false negative rates of the nonculture based techniques (15/95, 15.8%) and the swab-to-swab variation noted in the detection of culture itself (6/33, 18.2%), and the false negative specimens in both studies were associated with patients that appear to have low titer chlamydial infections.

Nonculture false positive results consist of true infections (culture failures) and real false positive results. The false positive nonculture test is the major concern regarding the substitution of rapid nonculture methods in place of the nearly 100% specific tissue culture

technique (125,129). A real false positive test result can lead not only to inappropriate antibiotic treatment of the patient, but also to inappropriate sexual contact tracing. This may lead to severe psycho-social consequences for the patient and/or the patient's family.

In this study, as explained in "Materials and Methods", (1) nonculture test false positive specimens were retested up to two times until a consistent result was obtained; if a positive nonculture test was repeatedly negative on two subsequent runs, the specimen was considered a true negative and the initial false positive result attributed to inter-run variability; (2) when ELISA and NIP tests remained false positive after repeat testing, their corresponding residual 2-SP specimens were tested (sometimes ELISA residual buffer was also tested) by DFA for elementary bodies. If elementary bodies were found in either residual transport medium, it is highly likely that the nonculture false positive tests were true chlamydial infections missed by culture. It has been suggested that some of these culture misses may actually be due to dead organisms present after antimicrobial therapy that are detected by the nonculture methods (95,125). In this study, however, it is unlikely that antimicrobial therapy was responsible for false positive nonculture results. Of those patients that had repeated false positive results, there were only two patients (one ELISA-only and one NIP-only) that had received antibiotics within one month preceding

their enrollment in the study. Those nonculture false positive tests that repeated positive, in which elementary bodies could not be detected in corresponding transport medium, are inconclusive cases because discrepancy analysis could not distinguish true chlamydial infections from actual false positive results.

In this study, six of 10 ELISA-only false positive specimens were due to inter-run variation, two were due to culture failures, in one case specimens were unavailable for discrepancy analysis, and one remains an unexplained false positive after analysis. Twenty-two of 49 NIP-only false positive specimens were due to inter-run variation (this method was still in development at the time of this study). Of the remaining 27, 22 correlated with either bloody specimens or clumping of specimens during processing (interferences which now appear to have been substantially reduced). One of the bloody specimens may, however, be a culture failure (elementary bodies were found in 2-SP). This leaves only five unexplained NIP false positive results. All four patients that were both ELISA and NIP false positive, appear to be due to culture failures (elementary bodies were found in 2-SP).

Three of the five unresolvable NIP tests are most likely real false positive results due to some unobservable material (blood or clumping not observed) in the specimen causing a false positive chemiluminescent reaction. This reasoning is supported by the observation that the IP method

which uses the same DNA probe as the NIP, but with a different label, was negative when tested on all three specimens. The other two unresolvable NIP specimens were also IP positive (positive with two different labels without clumping of magnetic spheres being noted) and may, in fact, be true infections that were not detected by culture or ELISA, or may be actual false positive results, possibly due to nonspecific binding of the probes to some material bound to the magnetic spheres. It is even remotely possible that the DNA probes are specifically binding to another organism's rRNA complementary base pair sequence, although the manufacturer has not found other organisms to which the DNA probes cross hybridize. Unfortunately, the DNA probe transport medium lyses cells, so the medium cannot be examined for chlamydial elementary bodies by DFA. In only one of the two ELISA-only unresolved positive patients could DFA discrepancy analysis be performed. The one unresolved ELISA positive may be a true chlamydial infection, but this is unlikely since elementary bodies were not detected in the patient's corresponding 2-SP specimen or in the ELISA's own specimen. This ELISA test is probably a real false positive result and, as reported by others (119,151), may be caused by crossreactivity of the ELISA's polyclonal antibodies with another gram-negative organism. In this study, crossreactivity of ELISA with other gram-negative organisms does not appear to be a significant problem as suggested by Taylor-Robinson and co-workers (151). Only 1/869 ELISA

specimens (.12%) was even possibly affected by crossreactivity. One patient with a false positive ELISA had to be excluded from this fraction because discrepancy analysis could not be completed in her case.

Tissue culture was the most sensitive and specific method used to detect Chlamydia. However, after correcting for what appear to be false negative cultures, the sensitivity of tissue culture fell to 93.1%, just slightly more sensitive than ELISA (90.2%), NIP (88.2%) and IP (85.3%). When considering the results of swab-to-swab variation in the culture variation study and number of potential culture misses in this study, the sensitivity rate of culture versus the nonculture methods are essentially equal in the populations studied. The advantage that culture maintains over the rapid nonculture tests is its 100% specificity rate, although ELISA and IP corrected rates are just slightly below at 99.0% and 98.4% (NIP is significantly lower at 93.8%).

The tissue culture procedure is costly, time consuming (2-6 days), and technically difficult to perform. Culture is, therefore, not performed in most laboratories. All three nonculture procedures, while still expensive, are easy to perform, are rapidly completed, and the results are determined using objective endpoint detection interpreted by instruments. The IP procedure does have the disadvantage of using a radioactive label. The IP test kits also have a short shelf life and are consequently limited to high volume

laboratories that are licensed for and use radioactive isotopes routinely.

The results of this study indicate that, in the populations studied, both sensitivity and specificity values of ELISA were acceptable for routine use when compared with culture. This is also true for the IP procedure, but its sensitivity appears to be slightly lower. Although the NIP procedure has nearly identical sensitivity to the ELISA procedure, its specificity was significantly lower. The NIP procedure was still in development when it was evaluated in this study. Using the manufacturer's updated NIP kit, 21/22 repeatedly false positive specimens due to gross blood and clumping of magnetic spheres tested negative. Further studies are required to insure that the manufacturer's reagent and method changes significantly curtail false positive reactions due to (1) gross blood, (2) clumping of magnetic spheres, and (3) inter-run variation. If the reagent changes have reduced these sources of false positive results to the levels seen in ELISA, the NIP test would also be acceptable for routine use.

The rapid nonculture techniques can play an important role in increasing detection of C. trachomatis infections, and thereby prevent the morbidity associated with complications. It is, however, most important that laboratory personnel and clinicians realize the limitations of the nonculture methods, especially in low prevalence populations (12,125,129). It may often be prudent to rerun

positive nonculture tests to rule out the possibility of inter-run variability causing false positive results. Also, in cases where a positive nonculture test appears to be questionable or may have medical-legal complications, it would be advisable to rerun the nonculture test and to include a confirmatory culture (2).

In summary: (1) there is no significant difference in the true positive rates (sensitivities) of ELISA, NIP, and IP as compared to tissue culture in either population; (2) in the populations studied, there was no difference between the false negative rates of the nonculture-based techniques and the swab-to-swab variation in detection of culture itself; (3) false positive results due primarily to inter-run variability, but secondarily to those which do not resolve (culture failures or real misidentifications), remain a problem with all three nonculture based techniques, (this phenomenon is more important in low prevalence populations where the predictive value of a positive test may be low and unacceptable); (4) there was a statistical difference in the true negative rates among the three nonculture methods; NIP had significantly more false positive results than ELISA or IP; most of these false positive results can be attributed in turn to three factors: (A) inter-run variability, (B) grossly bloody specimens, and (C) persistent clumping of magnetic spheres. Technical alterations have been made to address these problems and appear to have already solved at least the last two

problems; further studies are, however, required to insure that inter-run variability has been cut to a reasonable level (i.e. that of ELISA and IP); and (5) based on their sensitivities and specificities, the ELISA and IP methods could be used routinely in the populations studied; although the NIP technique was still in development, it appears to be reaching a performance level comparable to IP and the established ELISA method.

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PRESENTATIONS

Putbrese, S.C. (Invited Speaker). "Culture Variation in the Detection of Chlamydia trachomatis". Pathology Grand Rounds, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, 1988.

ABSTRACTS PUBLISHED

1. Putbrese, S.C., F.A. Meier, B.A. Johnson, R.R. Brookman, and H.P. Dalton. 1988. Comparison of a isotopic DNA probe and ELISA for detecting Chlamydia trachomatis directly in urogenital specimens. Annual meeting Interscience Conference on Antimicrobial Agents and Chemotherapy, Los Angeles, California, Abstract (to be presented).

2. Putbrese, S.C., F.A. Meier, B.A. Johnson, R.R. Brookman, and H.P. Dalton. 1988. Comparison of a non-isotopic DNA probe and ELISA for detecting Chlamydia trachomatis in clinical samples. Annual meeting American Society for Microbiology, Miami, Florida, C-234 Abstract.

3. Putbrese, S.C., F.A. Meier, B.A. Johnson, R.R. Brookman, and H.P. Dalton. 1988. Culture variation in the detection of Chlamydia trachomatis (C.t.). Annual meeting Society of Armed Forces Medical Laboratory Scientists, Reno, Nevada, 17:37 Abstract.